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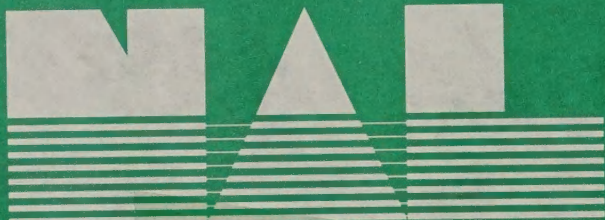
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**Cultivation and Detection of Mycoplasmalike Organisms**

USDA Competitive Grant No. 87-CRCR-1-2372

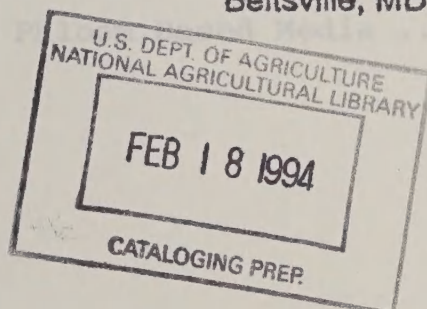
Final Report, July 1, 1991

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## ABSTRACT

Mycoplasma-like organisms (MLOs) cause hundreds of diseases worldwide, including many that damage crops in Israel and the United States. Nevertheless, the organisms have not been cultivated. In this cooperative project, advances were made that have led to improved detection of MLOs, to an improved understanding of their physiology, and to prospects for their eventual cultivation. Monoclonal antibodies were prepared against New Jersey aster yellows MLO, maize bushy stunt MLO, and the eastern X-disease MLO. Polyclonal antibodies and DNA probes were prepared against the walnut witches' broom MLO. Another group of plant/insect mollicutes (spiroplasmas) were used as models to study organismal physiology. In collaboration with D. Pollack, lysates of spiroplasma cells were examined to determine metabolic pathways. Of 67 enzyme activities studied, most were highly conserved within the genus. Thirty cultivation trials for several MLO species, utilizing generations of media from past concepts of mollicute physiology, were negative. However, by monitoring cultures with monoclonal antibodies and DNA probes, some evidence was obtained for brief flushes of growth in early culture intervals. The apparent presence of antigens common to MLOs and achleoplasmas, while concordant with recent placement of MLOs close to achleoplasmas on the basis of molecular phylogenetic evidence, were more likely procedural artifacts. Cultivation through seven passages of a previously uncultivable procaryotic nematode pathogen was obtained with media rationales deduced from MLO culture trials. The physiological data obtained in this study with other plant/insect mollicutes should lead to improved formulations tailored toward existing mollicute pathways and transport systems. Compilations of existing knowledge of MLO medium components, of mollicute physiology, and a detailed summation of reported MLO diseases worldwide were prepared.





## OBJECTIVES

1. Develop an assay system using monoclonal or polyclonal antibody for detection of the aster yellows MLO (AY-MLO) in cultures. [Note: Since we also developed gene probes, their development is included as a subobjective in this report.]

A. Develop monoclonal antibody techniques for monitoring AY-MLO growth during cultivation attempts and for purification of MLOs to be used for production of polyclonal antibodies.

B. Develop gene probes for monitoring AY-MLO growth during cultivation attempts.

2. Develop media for cultivation of the AY-MLO.





## INTRODUCTION

Several members of the Class Mollicutes (wall-less prokaryotes: Razin and Freundt, 1984) are important plant pathogens, causing hundreds of millions of dollars in damage on a worldwide scale. These pathogens can be essentially divided into two groups: (i) helical, motile, cultivable spiroplasmas, and (ii) somewhat spherical, nonmotile, noncultivable mycoplasmalike organisms (MLOs), the "yellows" agents and their allies (McCoy et al., 1989). Both multiply in their plant hosts and leafhopper vectors. The MLOs alone cause disease in more than 100 plant species, including many crop plants, and cause major crop losses in the United States. Although the yellows diseases were recognized as early as 1902, it was 1967 before a team of Japanese researchers (Doi et al., 1967) showed that plants afflicted with yellows diseases (e.g., mulberry dwarf or aster yellows) consistently contained mycoplasmalike organisms in their sieve tubes and that remission of disease symptoms in plants could be induced by tetracycline antibiotics (Ishiie et al., 1967). These discoveries prompted interest in MLOs as microbes and resulted in many attempts to cultivate them. While sporadic claims of cultivation of some of the agents have been made, other laboratories have been unable to repeat the results (Saglio and Whitcomb, 1979; Bové, 1984). Thus far, no claim has been made of fulfilling Koch's postulates, and none of the presumptive isolates has been deposited in any type culture collection.





Although it is possible that some of these organisms were MLOs which subsequently failed to multiply continuously in vitro because of inadequate culture media, others have been assumed to be common plant surface contaminants, such as Acholeplasma laidlawii.

Since culture attempts have been unsuccessful and only cumbersome bioassays were previously available for determining MLO presence or for monitoring their growth, microbiological research on these pathogens had essentially ceased in recent years. Two recent advances in mollicute research have now brought the study of yellows disease agents into a new era. These are: (i) development of monoclonal antibodies specific for MLOs (Lin and Chen, 1985), and (ii) development of gene probes to detect mollicute infection in plants or insects (Kirkpatrick et al., 1987). Successful cultivation of intracellular, fastidious mollicutes such as the sex-ratio organism (SRO) of Drosophila in insect cell culture (Hackett et al., 1986) and development of sensitive means for monitoring progress in cultivation of MLOs together created an opportunity for renewed attempts at cultivation of these plant disease agents. With the exception of a few fastidious spiroplasmas, success in cultivation of spiroplasmas has, indeed, been quite different than with the MLOs. All known plant-associated spiroplasmas are cultivable, and Koch's postulates have been fulfilled. One, Spiroplasma citri (Saglio et al., 1973), the causative agent of citrus stubborn disease, is a serious threat to citrus production





in the United States. It has been cultivated, characterized, and has now being used in a large number of basic microbiological studies (Bové et al., 1989). Similarly, the destructive corn stunt spiroplasma (S. kunkelii) was cultivated (Chen and Liao, 1975; Williamson and Whitcomb, 1975). A third plant spiroplasma, S. phoeniceum, has been similarly cultivated and characterized (Saillard et al., 1987). Although studies of spiroplasma nutrition and physiology have progressed steadily during the past 10 years, much remains to be done.



**OBJECTIVE 1A.** Develop monoclonal antibody techniques for monitoring AY-MLO growth during cultivation attempts, and for purification of MLOs to be used for production of polyclonal antibodies.

I. Development monoclonal antibodies against MLOs.

#### EXPERIMENTAL PROCEDURES

Diseased plants and insect vectors. For the aster yellows agent (AY-MLO), lettuce plants infected with the New Jersey strain of asters yellows (NJ-AY) were used. The AY-MLOs were transferred from infected to healthy plants by the leafhopper Macrosteles fascifrons.

The maize bushy stunt agent (MBS-MLO) was kept in corn (Zea mays cv. Golden Bantam Cross). The leafhopper vector Dalbulus maidis was used for transferring the MLO from plant to plant.

Celery plants infected with the Harrow isolate of EX-MLO (Eastern X-disease) were used. The leafhopper vector Paraphlepsius irroratus was used to transmit the disease.

Antigen preparation. Partially purified MLOs were obtained either from MLO-carrying leafhopper vectors (e.g., AY-MLO) or from diseased plants. MLOs from leafhoppers were prepared according to the method of Lin and Chen (1985). Approximately 2000 inoculative leafhoppers (M. fascifrons) were allowed to feed on AY-infected lettuce for 3 days and subsequently were maintained on rye plants for 2 weeks. Dissected salivary glands were homogenized in 0.02M phosphate buffered saline (PBS), and clarified by centrifugation (2000g). The supernatant was used as antigen.

Antigens were prepared from diseased plants following the methods of Jiang and Chen (1987). Briefly, diseased corn plants showing MBS symptoms were harvested, rinsed thoroughly in tap water, and cut into approximately 2.5 cm pieces, which were then macerated in PBS (Jiang and Chen 1987), using the slow and gentle cycle of a tissue homogenizer (Omni-mixer, Sorvall) until most parenchyma tissue was broken. The macerated tissues were strained through a mesh screen and retained vascular tissues were placed in the isolation medium and subjected to further maceration by mortar





and pestle. Extracted materials were centrifuged at 2600 X g for 6 min and the supernatant was centrifuged again at 27,000 X g for 30 min. The pellets were resuspended in a suspending medium. Then 0.6 ml was layered over each of several tubes containing step gradients, prepared from 15, 30, and 60% iso-osmotic Percoll (Pharmacia Inc., Uppsala, Sweden). Discontinuous density-gradient centrifugation was carried out at 14,000 X g for 29 min. Antigen was collected near the interphase between 30 and 60% Percoll layers.

Immunization. Six-week-old female BALB/c mice were immunized with partially purified MBS MLOs. The protein content of the antigen preparation was determined to be 260 ug/ml (Bio-Rad protein assay; Bio-Rad Lab., Richmond, CA). Antigen (0.15 ml) was mixed with an equal volume of Freundt's complete adjuvant and was then administered by intraperitoneal injection into each mouse once a week for 4 weeks. On days 35 and 36 the same amount of antigen was used for intravenous injections, except adjuvant was not added. The mice were sacrificed on day 38.

Monoclonal antibody selection. The procedure for splenic cells and fusion with myeloma cells (p3/NS1/1-Ag4-1) was the same as that described by Lin and Chen (1985). Hybridomas grown in HAT selective medium + 15% fetal bovine serum supplemented with  $10^{-4}$  M hypoxanthine,  $4 \times 10^{-7}$  M aminopterin, and  $1.6 \times 10^{-5}$  M thymidine) were screened for MBS MLO specific antibody production by using indirect ELISA with biotinylated antimouse immunoglobulin (Lin and Chen, 1985). ELISA plates were coated with preparations from both diseased and healthy corn plants (in 0.05 M carbonate buffer, pH 9.5) as described in Antigen Preparation, except that these preparations were not subjected to density-gradient centrifugation. Hybridomas that produced antibodies reacting with diseased and not with healthy plant preparations were selected for further cloning by limiting dilution. Selected monoclonal antibody producing hybridoma cells were subcultured for antibody production and the cell lines were stored in liquid nitrogen.

## RESULTS

1. Aster yellows. Seven monoclonal antibodies against NJAY MLO were obtained. These antibodies proved to be very specific and to possess high affinity. They do not react with the agents of eight other MLO-induced diseases including the Western strain of aster yellows.





2. Maize bushy stunt MLO. Twenty-six hybridoma clones, producing antibodies specific to MBS-MLO but not healthy corn plants, were selected. A total of 48 monoclonal antibodies were obtained.

3. Eastern X-disease MLO. Eighteen hybridomas secreted antibodies that reacted only with diseased celery plants but not with healthy celery preparations and were therefore specific to EX-MLO. The monoclonal antibodies produced by these hybridomas did not cross-react with preparations from plants infected with other MLO diseases. However, the monoclonal antibodies to EX-MLO reacted with preparations from celery infected with 3 strains of Western X-disease.

## II. Purification of MLOs.

1. Purification of MLOs from AY-infected lettuce. A technique was developed to partially purify MLOs associated with AY disease. Young lettuce plants were inoculated with the AY agent by leafhopper transmission and kept in a greenhouse. Leaf veins from symptomatic lettuce were isolated and homogenized in an isolation medium consisting of D-mannitol, 3-(N-morpholino)-propane sulfonic acid (MOPS), polyvinylpyrrolidone, L-cysteine, and ethylenediamine tetraacetic acid, before low (8 min at 1,500 X g) and high (30 min at 35,000 X g) speed centrifugation. The pellet was resuspended in medium containing mannitol and MOPS and layered on a discontinuous Percoll density gradient of 15,



30, and 50% (v/v). After centrifugation at 20,000 X g for 20 min, the fraction formed at the interface of the 30 and 50% solutions was collected and diluted with suspending medium. Gradient material was removed by centrifuging the diluted fraction at 100,000 X g for 2 hr. Electron microscopy of thin sections made from purified preparations showed an extremely high concentration of MLOs with well-preserved cellular structures identical to those in the phloem of diseased plants. These MLO cells exhibited a trilaminar limiting membrane, cytoplasmic ribosomal granules, and a network of DNA.

2. Purification using affinity chromatography. Purification of AY-MLO from diseased lettuce using monoclonal antibodies in an affinity chromatography procedure was developed. The affinity column consisted of Staphylococcus protein A covalently linked to a 6MB Sepharose matrix and coupled with monoclonal antibody specific against AY-MLO. AY-MLO was concentrated from clarified crude sap of diseased lettuce by differential centrifugation. After low-speed centrifugation, supernatants containing AY-MLO were loaded on the affinity column. Extraneous unbound plant materials from the crude sap were washed from the column after an incubation period. The aster yellows agent retained in the column was released by mechanical shaking and then eluted. The entire procedure took 3 hr. Intact and undamaged cells were observed by electron microscopy, and the high purity of this preparation was revealed by electrophoretic protein profiles and





the production of polyclonal AY-antiserum. In addition, the specificity of the monoclonal antibodies was confirmed by Western blots.

III. Production of polyclonal antibodies (PABs) using AY-MLO purified from affinity chromatography.

## EXPERIMENTAL PROCEDURES

Preparation of purified AY-agent. AY-MLOs were concentrated from clarified sap of diseased lettuce by differential centrifugation. Monoclonal antibody (MAb) against AY-MLO was coupled to protein A-Sepharose 6MB in an acrylic plastic column and incubated 20 min at room temperature. After washing, the MAb-conjugated protein A-Sepharose column was loaded with AY-concentrated crude extract and incubated. Unbound materials were washed from the column and bound AY-MLO was separated from MAb by mechanical shaking and then eluted from the column. The AY-MLO was pelleted, resuspended in PBS and used as antigen.

Preparation of polyclonal antiserum. BALB/c mice were immunized with the AY-MLO purified from the affinity column. Each mouse was subjected to 3 intraperitoneal injections 10 days apart. Each injection contained 100 mg AY-MLO protein mixed with an equal volume of Freund's complete adjuvant. Mice were bled 5 days after a final injection containing 10 mg of AY-MLO protein in saline.

## RESULTS

Immunoadsorption and electron microscopy. Electron micrographs of thin sections from pellets obtained after affinity chromatography from a diseased plant showed a very high concentration of AY-MLOs, indicating that the monoclonal antibody-coupled affinity column was an efficient instrument for trapping intact cells of the organism. MLOs of various sizes and shapes were recovered. Most of the recovered cells maintained





their cellular integrity, showing little or no damage. These cells exhibited a typical plasma membrane, ribosomal granules, and a fibrous network of DNA. No MLOs were found in eluates prepared from healthy control plants.

ELISA with polyclonal AY-antiserum. Previously it was found that polyclonal antisera from mice immunized with partially purified AY-MLO failed to distinguish between diseased and healthy lettuce plants because the sera cross-reacted with healthy plant proteins. In this study, the specificity of polyclonal AY-antiserum was increased with the use of highly purified AY-MLO antigen. In ELISA, antiserum discriminated between diseased and healthy lettuce plants.  $A_{490nm}$  values of diseased plants were about 7 times that of healthy plants. Similar results, but with much higher  $A_{490nm}$  values, were obtained with monoclonal antibody at comparable dilutions. The titer of polyclonal antiserum decreased with increasing dilutions.

IV. ELISA with polyclonal antisera against walnut witches' broom MLO.

#### EXPERIMENTAL PROCEDURES

MLO source. Leaves with severe symptoms of WWB were harvested from trees on the campus of Georgia Experiment Station at Griffin, Georgia.

MLO purification. The procedure of Hobbs et al. (1987) for MLO purification was used with minor modifications (Konai and



Chang, 1990).

Production of polyclonal antiserum. One ml of the partially purified preparation from walnut witches' broom infected or healthy leaves was mixed with 1 ml of Freund's incomplete adjuvant and injected intramuscularly into the hind leg of a New Zealand white rabbit. Five weekly injections were followed by five intravenous booster injections beginning 2 weeks after the final intramuscular injection. Bleedings were done 2 weeks after the last intramuscular injection and at weekly intervals before boosters.

Cross-adsorption of antiserum. WWB-MLO antiserum was cross-adsorbed at a 1:1 ratio with healthy walnut immunogen (Hobbs et al., 1987).

Preparation of gamma globulin and enzyme-conjugate. The procedure of Clark and Adams (1983) was modified for gamma-globulin purification (Konai and Chang, 1990). To prepare enzyme-conjugate, 1.0 ml of gamma globulin preparation was mixed with 2 mg alkaline phosphatase (P-5521, Sigma) and dialyzed in PBS pH 7.4 for 24 hr (with at least 4 changes) at room temperature. Glutaraldehyde (0.06% final concentration) was then added to the buffer and dialysis continued for an additional 4 hr. Excess glutaraldehyde was removed by dialysis for at least 24 hr. BSA at a final concentration of 5 mg/ml was added to the dialyzed enzyme-conjugated gamma-globulin and stored at 4° C.

ELISA procedure. The ELISA procedure of Clark and Adams (1983) was used with certain modifications (Konai and Chang, 1990).

## RESULTS

Dilutions of the coating and enzyme-conjugated gamma-globulin at 1:800 and 1:200, respectively, were able to detect the WWB-MLO in preparations of diseased walnut tissue (Table 1). WWB-MLO was detectable in infected fresh leaves, petioles and roots of walnut trees as well as frozen leaves and petioles. The MLO was also detected in leaves of grafted seedlings that displayed WWB symptoms in the greenhouse. ELISA readings from the leaves of graft-infected seedlings and field-infected walnut trees were similar. Readings of healthy leaves from the field or greenhouse





were much lower than readings of diseased leaves. Readings from healthy leaves were slightly higher than those of buffer controls, but were close to those from healthy petioles.

Table 1

Samples collected from infected and healthy walnut trees during the fall were frozen, and later tested for the presence of WWB-MLO. In these tests, leaves and petioles were tested separately. In each case, ELISA values were significantly higher than those of healthy controls (Table 2). Average ELISA values from frozen samples, from fresh leaves of infected walnut leaves, and from grafted walnut seedlings were within the same range (0.54 to 0.60), although individual readings from each leaf source varied (0.40 to 0.75). In further experiments, there was no significant difference (0.544 versus 0.49) in average ELISA values from symptomatic and asymptomatic leaves of the same WWB-infected tree. In this case as well, individual asymptomatic leaves gave



different readings (0.38 to 0.59). Healthy leaves gave average values close to the buffer control.

Table 2

To study the reactions of WWB-MLO with other mollicutes or presumed mollicutes, peach Western X-infected celery and aster yellows-infected periwinkle were tested. Peach Western X-infected celery gave a strong positive reaction to WWB-MLO. The highest reading was obtained with infected roots, followed by infected leaves. However, there was no difference in readings between infected and healthy celery petioles (Table 3). None of the other organisms tested, including aster yellows, pecan bunch MLO, and Spiroplasma citri showed positive reactions to WWB-MLO.

Table 3





**OBJECTIVE 1B.** Develop gene probes for monitoring AY-MLO growth during cultivation attempts.

## EXPERIMENTAL PROCEDURES

Isolation of walnut witches' broom MLO DNA. Leaves showing severe witches' broom symptoms and healthy leaves were harvested in Georgia in late fall of 1989 and stored in a freezer until used. Frozen leaves or petioles were freeze-dried and ground into powder before they were used for DNA extraction. About 0.5 to 1 g of tissue powder was added to 20 ml preheated CTAB buffer immediately after the addition of 0.5 ml mercaptoethanol. The extraction mixture was incubated at 65° C for 1 hr with occasional inversion, before an equal volume of chloroform-isoamylalcohol (24:1) was added. The components were mixed by inversion for 15 min before centrifugation at 6,500 rpm for 5 min. The pellet was washed in 500 ul 70% alcohol and transferred to a 1.5 ml Eppendorf tube. DNA was pelleted by centrifugation at 14,000 rpm for 5 min. The pellet was dissolved in 400 ul TE buffer. DNA was centrifuged in a CsCl gradient (Refractive Index = 1.3925) with 0.01 vol of 1 mg/ml bisbenzimidazole at 42,000 rpm (Beckman SW55 Ti) for 48 hr. MLO DNA was obtained by piercing the tube wall. The DNA was extracted twice with isopropanol, incubated at -20°C overnight and centrifuged at 14,000 rpm for 15 min. The DNA pellet was dissolved in 20 ul TE.

Cloning of WWB MLO fragments. WWB MLO DNA and pUC18 DNA were digested with HindIII and EcoR1 overnight before they were ligated with T4 ligase. The ligated DNAs were transformed into E. coli JM 83. WWB MLO DNA clones were screened with [<sup>32</sup>P]-labeled cloned plasmids.

## RESULTS

Comparison of WWB MLO with other MLOs. The WWB MLO was compared with other plant disease inducing agents. Using dot hybridization and Southern blotting techniques, at least one of the WWB MLO DNA clones (pWWB14 - 1.9 kb) hybridized strongly to DNA extracted from tissues of WWB infected trees, but only weakly to tissues from pecan bunch MLO infected trees, and not at all to blots of healthy walnuts and pecans or to periwinkle infected



with Western X, BLTVA, eastern and severe western aster yellows MLOs, and Spiroplasma citri. The presence of two to three visible bands, ranging from 0.9 to 5 kbp, in agarose gels after electrophoresis of WWB MLO DNA, indicated that WWB MLO may contain plasmid-like DNA.

**OBJECTIVE 2.** Develop media for cultivation of AY-MLO.

## EXPERIMENTAL PROCEDURES

MLO sources. The following sources of inoculum were used in MLO cultivation trials (Beltsville, University of Georgia, Michigan State University, Canada, and UC-Davis), gene probe development (UC-Davis and University of Georgia), and antibody production (Rutgers): New Jersey aster yellows (NJAY) MLO-infected Macrosteles fascifrons 5th instar-adults; Eastern aster yellows (EAY) MLO-infected M. fascifrons adults (Canada); beet leafhopper transmitted virescence agent (BLTVA)-infected beet leafhoppers (UC-Davis); potato yellow leaf roll-infected leafhoppers (from A. Purcell, UC-Berkeley); NJAY-MLO-infected aster plants (Beltsville and University of Georgia), severe aster yellows-, BLTVA-, or potato yellow leaf roll-infected celery plants (UC-Davis); NJAY-MLO-infected lettuce plants (Rutgers and Beltsville); frozen NJAY-MLO-infected leafhoppers or aster laminae or petioles (Beltsville), pecan bunch and walnut witches' broom infected leaves (University of Georgia), and *Oenothera*-infected leaf tip tissue cultures (Michigan State University). [The Beltsville strain of the soybean cyst nematode actinomycete (Pasteuria) sp. was used.]

Extraction temperature and atmosphere. Extraction was usually done at ambient temperature, although mortar and pestles were usually kept on ice. For three experiments (25-26, 30), extractions were done in a cold room with mortar and pestles kept on ice. Since oxygen may be harmful to MLOs, some extractions were performed in totally anaerobic environments. At the laboratory of J. Macy at UC-Davis, an anaerobic hood (Hood 1) with an atmosphere of 90% N<sub>2</sub>, 10% H<sub>2</sub> (28°C) was used. At the laboratory in Beltsville, an anaerobic hood (Anaerobe Systems) (Hood 2) with an atmosphere of 90% N<sub>2</sub>, 5% CO<sub>2</sub>, and 5% H<sub>2</sub> (26°C) was used.

Sterility: Antibiotics and filtration. To reduce the level of contaminants, antibiotics were generally added to the media (see media tables). In some cases, filtration through 0.45 or 0.6 um





Millex filters was employed (see Procedures below).

Extraction procedures. Extractions for production of gene probes (Kirkpatrick *et al.*, 1987) and monoclonal antibodies (Lin and Chen, 1985) have been described.

**Extraction procedures for MLO cultivation trials** (see Table 4) were the following:

Extraction procedure 1. Leafhoppers were placed in a state of torpor by cooling them for a few minutes in a freezer. 50 mg of infected *M. fascifrons* adults or diseased (0.1% sodium hypochlorite surface sterilized) plants were ground with a mortar and pestle in 1-4 ml extraction medium. Plant tissue was often diced before grinding and filtered through cheesecloth afterwards. All extracts were clarified by centrifugation in a clinical centrifuge for 5-10 minutes, and filtered through 0.45 (Expt. 1-7, 10, 27, 30) or 0.65 (Expt. 19-22)  $\mu$ m Millex filters. In Expt. 25-26, no filters were used. In Expt. 21, the ability of bacterial contaminants to pass through 0.22, 0.3, 0.45, 0.65, and 0.8  $\mu$ m Millex filters was compared.

Extraction procedure 2. This procedure was the same as Extraction Procedure 1, but a buffer was used rather than a medium. The extract was passed through 0.65  $\mu$ m Millex filters.

Extraction procedure 3. In this procedure (at Rutgers), NJAY-MLO-infected lettuce was cut, homogenized and strained through sieves to eliminate parenchyma tissue. Vascular tissue was then ground in the isolation medium, centrifuged twice in a clinical centrifuge, and purified using gradient centrifugation in 15, 30, and 50% Percoll. Purified MLOs were extracted from the interfaces.

Extraction procedure 4. At UC-Davis, approximately 30-50 infected leafhoppers were briefly frozen and (Expt. 13) ground in 3 ml of extraction medium with a glass tissue grinder or (Expt. 16) diced in 2 ml of medium in a glass plate. The resulting brei was added to 20 ml of extraction medium, centrifuged at low speed (3500 rpm) for 5 minutes, and stored on ice. After a high speed (11,000 rpm) centrifugation for 20 minutes, the pellets were resuspended in media and filtered through 0.45  $\mu$ m filters.

Extraction procedure 5. At UC-Davis, approximately 1 g of diseased celery tissue near the center of the plant (generally crown tissue) was cut into small pieces, placed in 500 ml 70% ethanol for 1.5 minutes to eliminate surface contaminants, and washed in distilled water. The celery tissue was then ground with a cold mortar and pestle in 10 ml of extraction medium, and the extract was added to 20 ml of BEM1, centrifuged at low speed



(3500-4000 rpm) for 5 minutes, and stored on ice. After a high speed (11,000 rpm) centrifugation for 20 minutes, the pellets were resuspended in media and filtered through 0.45 um filters.

Extraction procedure 6. At Ottawa, Canada, 50 EAY-MLO-infected *M. fascifrons* adults were ground, by L. N. Chiykowski, in extraction medium (1/10 dilution, w/v), centrifuged (3000 rpm) for 10 minutes, filtered (0.45 um), diluted to 1/50 in culture medium, divided into 0.2 ml portions, and stored in the refrigerator. Alternatively, 0.01 M PBS was used for extraction and dilution.

Extraction procedure 7. Finely drawn glass needles were used to collect hemolymph from approximately 50 MLO-infected leafhoppers. Immediately upon collection, the hemolymph was added to a small glass tube containing 50 ul of culture medium, and kept on ice.

Extraction procedure 8. These extractions were performed by B. Sears (Michigan State University) by a method (to be published later) that relied on plant leaf tip cultures for inoculum.

#### **Extraction media.**

]Extraction media are described in Tables 20-24. Three strategies were used: (i) media used for cultivation were used for extraction; (ii) buffers (e.g., PBS and BEM1-2) were used; and (iii) hybrid buffers/media with large amounts of reducing agents were used (e.g., MEMEK1, M1D-SO<sub>3</sub>, Percoll).

#### **Conditions of Cultivation.**

Temperature/atmosphere. In general, the same temperature and atmospheric conditions that were employed for extraction were used for cultivation. In addition, cultivation was attempted in a GasPak system (95% CO<sub>2</sub>, 5% H<sub>2</sub>) in Expt. 13. To test the viability of MLOs under cooler conditions, refrigeration (4°C) was used in Expt. 17-19; 15°Cf was used in Expt. 15. In Expt. 19, mineral oil was added to the tops of the wells to limit oxygen.

Type of culture vessel. Culture was attempted in 24-well tissue culture (microtiter) plates, 3 ml plastic tubes, or on solid media. In Expt. 4, the yeast co-culture was placed in a well insert that had a pore diameter of 0.4 um.

Media co-culture cells. Cultivation media were of two types, liquid or solid (0.8% agar), as described in Tables 20-24. In





Experiments 1-4, 8, 15, 20, and 27, co-culture was attempted, using a yeast obtained from D. L. Williamson (Stony Brook) that had been successfully used to isolate the sex ratio spiroplasma of Drosophila, bacteria isolated in our investigations, or insect cells obtained from D. L. Lynn (DU-E, Tex2) or H.-T. Hsu (AC-20).

Means of determining growth. Four methods were used to determine the amount of MLO growth in culture systems: (i) microscopic enumeration under a dark-field microscope at 1250x; quantification through (ii) antibody/ELISA systems or (iii) gene probe dot blots; and (iv) bioassay. Microscopic enumeration was used primarily for estimating counts of spiroplasmas and yeasts used in the culture trials. In this case, color changing units (CCUs) were also used. Structures were observed in MLO cultivation trials that may or may not have been MLOs; the morphologies and numbers of these cells were also recorded. Procedures for use of gene probes (Kirkpatrick et al., 1987) and antibody/ELISA (Lin and Chen, 1985) have been described. For gene probes, DNA was extracted by the alkaline lysis method (Maniatis et al., 1982) and the BRL HybriDot system was used for blots. rRNA probes were used at UC-Davis (Kirkpatrick et al., 1987). Probe pmp9 (a clone of the entire 4.2 kbp plasmid from the *Oenothera*-MLO was used by B. Sears at Michigan State University. For ELISA, samples (in EDTA and mercaptoethanol) were added to microtiter wells with a carbonate-bicarbonate, pH 9.6 coating buffer, and placed in a moist chamber at 37°C for 3 hours. 5% skim milk was used as a blocking solution. Washes were made with PBS-Tween 20. After addition of monoclonal or polyclonal antibody, biotinylated goat antimouse IgG and IgM conjugate (KPL) were added. Strepto-Avidin peroxidase conjugate (KPL) was added and reaction with horseradish peroxidase substrate was monitored by an ELISA reader. For bioassay, finely drawn glass needles were used to inject (approximately) 0.1 ul of MLO extract or culture between the sternites of 50-100

M. fascifrons 5th instar or adult leafhoppers. Insects were kept moist and cool until injection, which was performed under CO<sub>2</sub>. They were then placed on rye plants. After an incubation period of 10 days, and until at least 28 days, leafhoppers were placed on aster plants for MLO transmission; exposed plants were observed for symptoms of yellows disease.

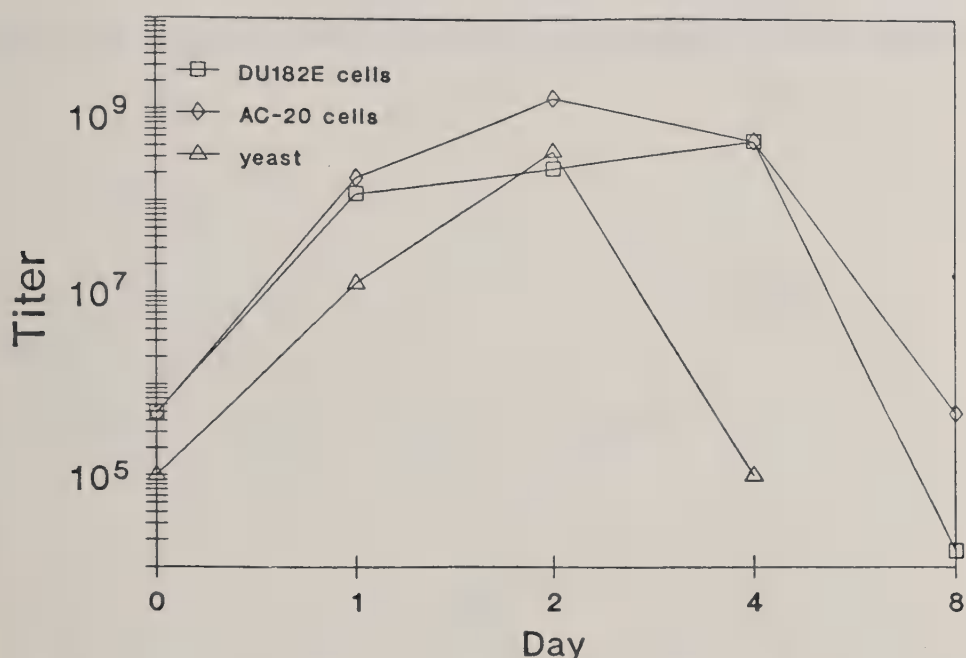
### **Spiroplasma Cultivation Experiments.**

Fastidious spiroplasmas. In an attempt to determine the impact of co-culture on nonfastidious (S. apis) and fastidious (S. kunkelii) spiroplasmas, as an index of what we could expect from MLOs in similar systems, we monitored spiroplasma growth in DCCM or AC-20M medium in co-culture with DU-E or AC-20 leafhopper cell lines, respectively, with or without yeast cells.



Experiment 11 - Spiroplasma apis. S. apis (strain B31) cultures in DCCM medium were added to microtiter wells containing DCCM medium and the DU-E beetle cell line, the AC-20 leafhopper cell line, or Williamson's yeast cells. Each well contained approximately  $7.5 \times 10^4$  insect cells, which were added fresh at the start of the experiment. Growth of spiroplasmas was monitored daily for 8 days by microscopic enumeration and color changing units (CCUs).

Experiment 12 - Spiroplasma kunkelii. The procedure was the same as for Experiment 11, but S. kunkelii (strain E275) cells were used.




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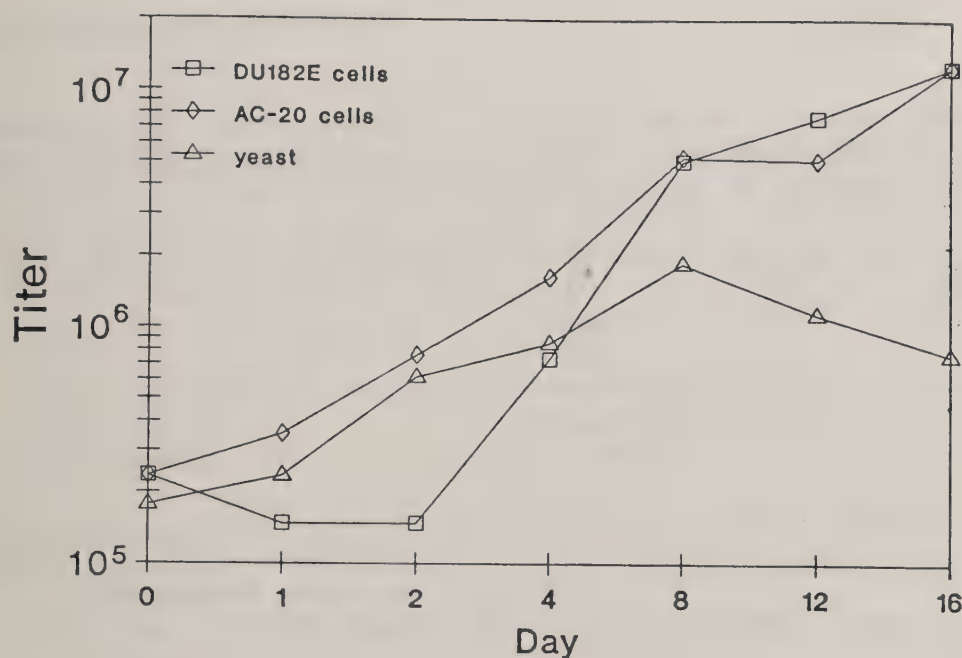
Fig. 1. Titers of S. apis in three cell-assisted media for MLO cultivation

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Comparison of Co-culture Systems. Our preliminary studies with Spiroplasma apis and S. kunkelii suggest (on the basis of analysis of microscopic enumeration data) that the nature of cells used in co-culture systems (DU-E or AC-20 insect cells, or yeast cells) affects mollicutes differentially. For S. apis



(Fig. 2), yeast > AC-20 > DU-E cells; for *S. kunkelii*, AC-20 > yeast > DU-E cells (where > = better growth). Since *S. apis* is a relatively fast growing species, it is not surprising that it is able to take advantage of fast-growing co-culture yeast cells. AC-20 co-cultures may be more suitable for slower growing mollicute cells, such as *S. kunkelii*. Since *S. kunkelii*, like the MLO, is a phloem/leafhopper-associated organism, and grew preferentially in the leafhopper-derived AC-20 co-culture system, this system may be superior to yeast systems for co-culture of




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Fig. 2. Titters of *S. kunkelii* in three cell-assisted media for MLO cultivation

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MLOs. However, it should be noted that a slow growing spiroplasma, the group II sex ratio organism of Drosophila, was more consistently isolated in yeast co-cultures than in insect cell (lepidopteran) co-cultures.

These results, when considered together, suggest that important principles may be revealed from continued attempts to cultivate other fastidious spiroplasmas or other mollicutes in cell lines from different sources

### **MLO Cultivation Experiments.**

#### **EXPERIMENTAL PROCEDURES**

Experiment 1 - Co-culture with DU-E insect cells and Colorado potato beetle spiroplasmas. DU-E cells were set up as in Expt. 11 (above). Cells were allowed to grow in the microtiter wells for one day prior to inoculation of MLO-containing extracts. Colorado potato beetle spiroplasmas (strain LD-1) were inoculated to a final concentration of  $2 \times 10^5$  cells per ml. 0.2 ml of inoculum was added to 1.8 ml of medium in each well. Treatments were:

- o DU-E Cells/LD-1 Cells/DCCM Medium/NJAY-MLO Extract from M.f.
- o DU-E Cells DCCM Medium/NJAY-MLO Extract from M.f.
- o DU-E Cells DCCM Medium/Healthy Extract from M.f.

Experiments 2-3 - Co-culture with DU-E beetle cells. The procedure was as described in Experiment 1; no LD-1 cells were used. Treatments were as follows:

- o DU-E Cells/DCCM Medium/NJAY-MLO Extract from M.f.
- o DU-E Cells/DCCM Medium/Healthy Extract from M.f.

Experiment 4 - Co-culture with AC-20 leafhopper cells. The procedure was as described in Experiment 1, except AC-20 leafhopper cells were used, and log phase yeast cells were placed in 0.4 um pore "Minicell" inserts in the wells. Treatments were as follows:

- o AC-20 Cells/AC-20M Medium/yeast/NJAY-MLO Extract from M.f.
- o AC-20 Cells/AC-20M Medium/yeast/Healthy Extract from M.f.



Experiments 5-7 - Culture in M1D. These experiments were designed to determine the rate of transmission of injected leafhoppers, using extracts in M1D medium, with the reducing agent  $\text{Na}_2\text{SO}_3$  added to the media used for extraction of MLOs from plants. Treatments were as follows:

- Expt 5: M1D/NJAY-MLO Injected Leafhoppers as Inoculum
- Expt 6: M1D- $\text{SO}_3$ /Fresh NJAY-Diseased Petioles as Inoculum  
M1D- $\text{SO}_3$ /Fresh NJAY-Diseased Laminae as Inoculum
- Expt 7: M1D- $\text{SO}_3$ /Shell-frozen NJAY-Diseased Petiole Inoculum

Experiment 8 - Co-culture with AC-20 leafhopper cells with MLO cells trapped on 0.45  $\mu\text{m}$  filters. In this experiment, MLOs were extracted in a buffer and filtered through overlying 0.65/0.45  $\mu\text{m}$  membranes. Presumably, most MLO cells would pass through the 0.65  $\mu\text{m}$  membrane and be retained by the 0.45  $\mu\text{m}$  membrane. The 0.45  $\mu\text{m}$  membrane was removed and placed in culture with AC-20 cells. The experimental set-up was similar to Experiment 1.

- AC-20 Cells/AC-20M Medium/0.45  $\mu\text{m}$ /NJAY-MLO Extract from M.f.
- AC-20 Cells/AC-20M Medium/0.45  $\mu\text{m}$ /Healthy Extract from M.f.

Experiment 9 - Percoll gradient purification and culture in C-3G/M1D media. For the purpose of obtaining high quality inoculum, lettuce was used to supply NJAY-MLOs extracted and purified on Percoll gradients. Because we suspected that Percoll, extraction medium, or contaminants might cause high mortality of injected leafhoppers, filtration through 0.45  $\mu\text{m}$  membranes was used in some trials. There were three parts to this experiment. In Parts A and C, enzymes were used to digest plant tissue, leaving only vascular material as the MLO source.

- A: 10-50% Percoll gradient/Extract from lettuce/C-3G Medium
- B: Extract from lettuce/C-3G Medium  
+/- Filtration  
+ 0, 40, and 100% Percoll
- C: Extract from lettuce/Percoll gradient (3 bands injected)

Experiment 10 - Survival and transmission efficiency of  $\sigma/\varphi$ 's. This experiment was conducted to determine the relative susceptibility and disease transmission potential of male and female leafhoppers. Since the extracts and resulting AC-20M cultures were tested for MLO survival, this was also a cultivation experiment.

- Injected  $\sigma$ 's: NJAY-MLO in AC-20M Medium  
AC-20M Medium Control  
Noninjected Control
- Injected  $\varphi$ 's: NJAY-MLO in AC-20M Medium  
AC-20M Medium Control  
Noninjected Control

Experiment 13 - BLTVA- & SAY-MLO culture in Chang medium under air or GasPak conditions. Experiments 13-16 were conducted by K. Hackett and B. Kirkpatrick at UC-Davis, in collaboration with C.-J. Chang, J. Macy, C. Kuske, M. Feldlaufer, D. Golino,





D. Williamson, and A. Purcell. In these experiments, cultures were monitored by gene probes and polyclonal antibodies developed at the laboratory of B. C. Kirkpatrick. During these experiments, anaerobic conditions were used for the first time. Most cultures were incubated at 28°C.

In Experiment 13, leafhoppers were used as a source of BLTVA-MLO and celery was used for SAY-MLO. All extractions were done in Kirkpatrick's extraction buffer (BEM1). Chang's medium was used. This medium, designed by C.-J. Chang, was based on celery extract. Treatments were as follows:

- o BEM1/BLTVA-MLO/Air
- o BEM1/BLTVA-MLO/GasPak
- o BEM1/SAY-MLO/Air
- o BEM1/SAY-MLO/GasPak

Experiment 14 - BLTVA- and PYLR-MLO culture in Chang based media under air or GasPak conditions. This experiment was similar to Experiment 13, but PYLR-MLO was used instead of SAY-MLO, and different extraction (BEM2) and culture (DMCMH) media were used. An anaerobic hood (Hood 1) was used instead of a GasPak. The DMCMH medium was extremely complex, and was a compilation of DCCM, M1D, Chang, H-2, and H-1 media. Treatments were as follows:

- o BEM2/BLTVA-MLO/Chang Medium/Air
- o BEM2/BLTVA-MLO/Chang Medium/Anaerobic Hood
- o BEM2/BLTVA-MLO/DMCMH Medium/Air
- o BEM2/BLTVA-MLO/DMCMH Medium/Anaerobic Hood
- BEM2/PYLR-MLO/Chang Medium/Air
- BEM2/PYLR-MLO/Chang Medium/Anaerobic Hood
- BEM2/PYLR-MLO/DMCMH Medium/Air
- BEM2/PYLR-MLO/DMCMH Medium/Anaerobic Hood

Experiment 15 - PYLR-MLO culture in P1, Chang, CBY, and M1D media, under air or anaerobic hood conditions, +/- yeast, plant extract inoculum. This experiment differs from the previous two in that additional media were used (P1, CBY, M1D) and, of possible importance, all extractions were done in completely anaerobic environments. Aliquots of each extract were then left in the anaerobic hood or removed to an aerobic incubator. Also, the medium to be used for culture was used in the extraction. Media used in these trials were very complex. CBY medium was based on Chang medium, with added fresh brain extract, extracted by B. C. Kirkpatrick. Yeast extract was obtained from C. Kuske. Pig brain extraction was obtained by using a tissue grinder to macerate neonate pig brains in cold 20 mM KCl/0.03 M HEPES buffer. The brei was centrifuged for 2 hours at 37,000 rpm, and the supernatant was filtered to remove contaminants. P1 medium was the first medium designed by us to mimic plant phloem sap, and contained aster lipid extracts obtained from M. Feldlaufer. It also contained complex components such as pig brain and yeast extract. Yeast co-culture was attempted in half of the trials.



The treatments were as follows:

- o PYLR-MLO/M1D Medium/Air
- o PYLR-MLO/M1D Medium/Air/Yeast
- o PYLR-MLO/M1D Medium/Anaerobic Hood
- o PYLR-MLO/M1D Medium/Anaerobic Hood/Yeast
- PYLR-MLO/Chang Medium/Air
- PYLR-MLO/Chang Medium/Air/Yeast
- PYLR-MLO/Chang Medium/Anaerobic Hood
- PYLR-MLO/Chang Medium/Anaerobic Hood/Yeast
- o PYLR-MLO/CBY Medium/Air
- o PYLR-MLO/CBY Medium/Air/Yeast
- o PYLR-MLO/CBY Medium/Anaerobic Hood
- o PYLR-MLO/CBY Medium/Anaerobic Hood/Yeast
- PYLR-MLO/P1 Medium/Air
- PYLR-MLO/P1 Medium/Air/Yeast
- PYLR-MLO/P1 Medium/Anaerobic Hood
- PYLR-MLO/P1 Medium/Anaerobic Hood/Yeast

Experiment 16 - PYLR-MLO culture in P1, Chang, CBY, and M1D media, under anaerobic hood conditions, leafhopper inoculum.

This experiment was similar to Experiment 15, except leafhoppers, obtained from A. Purcell, were used for PYLR inoculum. All cultures were maintained under the anaerobic hood. Treatments were as follows:

- o PYLR-MLO/Anaerobic Hood/M1D Medium
- PYLR-MLO/Anaerobic Hood/Chang Medium
- o PYLR-MLO/Anaerobic Hood/CBY Medium
- PYLR-MLO/Anaerobic Hood/P1 Medium

Experiments 17-18 - EAY-MLO culture in MIMEK1 and PBS. In Experiments 17 and 18, eastern (EAY) and western (WAY) strains of aster yellows were used by L. Chiykowski to compare survival of MLOs in the MEMEK1 extraction medium/MIMEK1 culture medium system of K. Hackett to survival in 0.01 M phosphate buffered saline. The MEMEK1 extraction medium was designed to protect MLOs from oxygen. MIMEK1 was a medium that was designed to minimize MLO membrane damage, but without the strong buffers and reducing agents of MEMEK1. Although leafhopper extractions were carried out at ambient temperatures, the cultures were immediately refrigerated for the duration of the experiments, which were 5 (Expt 17) and 4 (Expt 18) days. Treatments were as follows:

- o Expt 17: EAY/4°C/MEMEK1/MIMEK1
- o Expt 17: EAY/4°C/PBS
- Expt 18: WAY/4°C/MEMEK1/MIMEK1
- Expt 18: WAY/4°C/PBS

Experiment 19 - NJAY-MLO culture in PL-1 medium, under air or reduced oxygenation (by oil overlays), At 26°C or 4°C. Two innovations were introduced in this trial: a medium (PL-1) based on an extensive review of the literature on the composition of plant phloem sap, and through consultation with J. D. Pollack (Ohio State University) and J. G. Tully (NIH) (see Appendix 1);





reduced oxygenation through the use of mineral oil overlays on cultures in wells. The effect of refrigeration on MLO survival was also reevaluated. Treatments were as follows:

- o NJAY-MLO/26°C/Air
- o NJAY-MLO/26°C/Oil
- o NJAY-MLO/4°C/Air
- o NJAY-MLO/4°C/Oil

Experiment 20 - Co-culture of NJAY-MLO with bacteria. Previous evidence had shown that minute (unidentified) "satellite" bacterial colonies occurred on solid media during MLO isolation attempts. Because of the metabolite retaining properties of solid media (see Appendix 1), the possibility exists that MLOs might grow better in this milieu than in liquid media. To study these factors, we conducted the following series of experiments (Experiments 20-23). In all experiments, NJAY-MLO-infected M. fasciifrons was used for inoculum. Either PL-1 or PL-2 (a double strength PL-1) media were used for extraction.

In Experiment 20, Luria, blood, and milk agar plates were used for culture; PL-1 liquid medium with Na<sub>2</sub>SO<sub>3</sub> was used for extraction, and PL-1 liquid medium was used for culture. A bacterium (Bact1) isolated during Experiment 19 from NJAY-MLO-infected leafhoppers was used for one series of co-culture attempts (this bacterial culture was presumed to have some viable MLOs). Other bacteria (BacMx, used in Expt. 21) were obtained during isolation from NJAY-MLO-infected leafhoppers and plants. Extractions were similarly performed from healthy leafhoppers and plants (not shown in the treatment schema). Media were observed for bacteria and the presence of satellite MLO colonies.

Treatments were as follows:

- o Bact1 & NJAY-MLO/PL-1 Liquid Medium
- o Bact1 & NJAY-MLO/Blood Agar
- o Bact1 & NJAY-MLO/Milk Agar
- o Bact1 & NJAY-MLO/Luria Agar
- NJAY-MLO-Infected Leafhoppers/PL-1 Liquid Medium
- NJAY-MLO-Infected Leafhoppers/Blood Agar
- NJAY-MLO-Infected Leafhoppers/Milk Agar
- NJAY-MLO-Infected Leafhoppers/Luria Agar
- NJAY-MLO-Infected Plants/Luria Agar/Unfiltered Inoculum
- NJAY-MLO-Infected Plants/Luria Agar/0.65 um Filtered Inoculum

Experiment 21 - To Determine if cultured bacterial contaminants pass through filters with MLOs. Evidence from previous experiments had suggested that small bacteria might be passing through 0.45 um filters. If so, filtration, which is known to significantly reduce MLO inoculum titer (even through 0.65 um pore filters) (R. F. Whitcomb, unpublished data), would not be a practical method of obtaining adequate MLO inoculum for culture trials. This experiment is included here because NJAY-MLO culture was attempted during the study. Essentially, a mixed bacterial culture (BacMx; see Expt. 20), containing MLO extract,





was passed through 0.22, 0.3, 0.45, 0.65, and 0.8 um filters and plated on Luria agar.

- o NJAY-MLO/BacMx/Luria/Unfiltered
- o NJAY-MLO/BacMx/Luria/0.22 um Filtered
- o NJAY-MLO/BacMx/Luria/0.3 um Filtered
- o NJAY-MLO/BacMx/Luria/0.45 um Filtered
- o NJAY-MLO/BacMx/Luria/0.65 um Filtered
- o NJAY-MLO/BacMx/Luria/0.8 um Filtered

Experiment 22 - NJAY-MLO bacterial co-culture on solid medium under air and anaerobic hood conditions. This experiment was conducted, principally, to determine if MLOs might grow on solid medium under anaerobic conditions. Besides concentrating metabolites, solid media might act as a sieve trap for the limited inoculum. This experiment was also a co-culture experiment in that no antibiotics (and only 0.8 um filtration) was used in the extraction, which was done anaerobically. Penicillin discs were placed on the medium to provide a zone of bacterial inhibition, thus combining, in one experiment, the concepts of bacterial co-culture and bacterial-free isolation. Multiple PL-2A agar plates were used. Treatments were as follows:

- o NJAY-MLO +/- Bacteria/PL-2A/Air
- NJAY-MLO +/- Bacteria/PL-2A/Anaerobic Hood

Experiment 23 - NJAY-MLO culture on solid media, under air and anaerobic hood conditions, hemolymph inoculum. This experiment was similar to Experiment 22, but no bacterial co-culture was attempted; penicillin-G was used to eliminate bacterial contaminants on the plates (PL-2A) and in liquid medium (PL-2). And, for the first time in our experiments, hemolymph from infected insects was used as inoculum. This was done to provide high quality inoculum free of toxins that are released during extraction of plant and insect material, and to reduce bacterial contamination. Treatments were as follows:

- o NJAY-MLO/PL-2A Agar/Air
- o NJAY-MLO/PL-2A Agar/Anaerobic Hood
- o NJAY-MLO/PL-2 Medium/Air
- o NJAY-MLO/PL-2 Medium/Anaerobic Hood

Experiment 25 - NJAY-MLO culture from lettuce in serum-free and serum fraction media, under air and anaerobic hood conditions.

Experiments 25 and 26 were conducted to test two concepts. One was that lettuce, because of the high quantities of infected tissue that can be processed, might be a better source of inoculum than leafhoppers. The second was that, since MLOs may be closely related to acholeplasmas (Lim and Sears, 1989), typical acholeplasma media, such as serum-free (SerFree) and serum fraction (SerFrac) media, might be effective growth media for MLOs. Levels of Tween 80 (from 0-0.4%; SerFree 0 = 0%), found to be important for acholeplasma cultivation, were employed. All extractions were performed in the cold room to





reduce damage due to oxygenation. Cultures in both experiments were monitored by T.-A. Chen using monoclonal antibody/ELISA procedures developed during the course of this grant. Treatments were as follows:

- oo NJAY-MLO/Air/SerFrac Medium
- o NJAY-MLO/Air/SerFree0 Medium
- o NJAY-MLO/Air/SerFree1 Medium
- o NJAY-MLO/Air/SerFree2 Medium
- o NJAY-MLO/Air/SerFree4 Medium
- NJAY-MLO/Anaerobic Hood/SerFrac Medium
- NJAY-MLO/Anaerobic Hood/SerFree0 Medium
- NJAY-MLO/Anaerobic Hood/SerFree1 Medium
- NJAY-MLO/Anaerobic Hood/SerFree2 Medium
- NJAY-MLO/Anaerobic Hood/SerFree4 Medium

Experiment 26 - NJAY-MLO culture from lettuce in serum-free, PL-1, and M1D media, under air and anaerobic hood conditions.

In this experiment, other promising media (PL-1 and M1D) were used in addition to serum-free medium with 0.4% Tween 80.

Treatments were as follows.

- o NJAY-MLO/Air/SerFree4 Medium
- o NJAY-MLO/Air/PL-1 Medium
- o NJAY-MLO/Air/M1D Medium
- NJAY-MLO/Anaerobic Hood/SerFree4 Medium
- NJAY-MLO/Anaerobic Hood/PL-1 Medium
- NJAY-MLO/Anaerobic Hood/M1D Medium

Experiment 27 - NJAY-MLO co-culture with an insect muscle cell line. During the course of these experiments, D. Lynn, one of our BARD collaborators, developed a cell line that had not previously been tried in mollicute co-culture. The intriguing aspect of this cell line was that it was the first insect muscle cell line ever developed. Importantly, it does not multiply; thus it might provide the advantages of co-culture for a slow growing fastidious mollicute, without depriving the mollicute of nutrients [nutrient competition was shown to play a negative role in co-culture of fastidious spiroplasmas (Hackett *et al.*, 1986)]. To study this, the muscle cell line (Tex2) was set up in two different media, Tex2M medium, normally used to grow the cell line, and DCCM medium, which had previously been used to cultivate fastidious spiroplasmas. All culture was attempted aerobically. Inoculum consisted of cultures (Experiments 23 and 25) that had tested positive by ELISA for acholeplasmas or NJAY-MLOs, and fresh NJAY-MLO extract. Treatments were as follows:

- o NJAY-MLO-Leafhopper Extract/Tex2 Cell Line/DCCM Medium
- NJAY-MLO-Leafhopper Extract/Tex2 Cell Line/Tex2M Medium
- o Expt 23 Culture in PL-1 Medium/Tex2 Cell Line/DCCM Medium
- Expt 23 Culture in PL-1 Medium/Tex2 Cell Line/Tex2M Medium
- o Expt 25 Culture in PL-1 Medium/Tex2 Cell Line/DCCM Medium
- Expt 25 Culture in PL-1 Medium/Tex2 Cell Line/Tex2M Medium





Experiment 28 - Oenothera-MLO culture from explants in serum fraction and PL-1 media, under aerobic conditions.

In this experiment, serum fraction and PL-1 media were sent to B. Sears (Michigan State University) where they were inoculated with Oenothera-MLOs from explants. At that time, Sears also attempted cultivation in additional media variants formulated at Michigan. Treatments using Beltsville formulated media were as follows:

- Oenothera-MLO/Air/SerFrac Medium
- Oenothera-MLO/Air/PL-1 Medium

Experiment 29 - Oenothera-MLO culture from explants in PL-1M, serum fraction and PL3 series media, under aerobic conditions.

In this experiment, media were sent to B. Sears (Michigan State University), where they were inoculated with Oenothera-MLOs from explants. The PL3 media series was another attempt to mimic plant phloem sap. Using a (mostly) defined medium (PL3-2) as a base, undefined components (or additional defined components) were added to make variants. PL-1M medium was PL-1 medium with high levels of undefined components. Treatments were as follows:

- Oenothera-MLO/Air/SerFrac Medium
- Oenothera-MLO/Air/PL3-1 Medium
- Oenothera-MLO/Air/PL3-2 Medium
- Oenothera-MLO/Air/PL3-3 Medium
- Oenothera-MLO/Air/PL3-4 Medium
- Oenothera-MLO/Air/PL3-5 Medium
- Oenothera-MLO/Air/PL3-1M Medium
- Oenothera-MLO/Air/PL3-2S Medium
- Oenothera-MLO/Air/PL3-2Y Medium
- Oenothera-MLO/Air/PL3-2SY Medium
- Oenothera-MLO/Air/PL3-P Medium

Experiment 30 - Oenothera-MLO culture from explants in serum fraction, M1D, PL-1M, and PL4 series media, under aerobic conditions.

Because of increasing evidence that factors that affect membrane stability are important in MLO maintenance, the PL4 media series was designed to incorporate high levels of membrane stabilizers, at a wide range of osmolarities. These media were sent to C.-J. Chang (University of Georgia) and B. Sears (Michigan State University) where they were inoculated with NJAY- and Oenothera-MLOs, respectively. Data from the Georgia and Michigan work is not yet available. At Beltsville, inoculum consisted of extracts from NJAY-MLO infected leafhoppers. All extractions were performed in the cold room to reduce damage due to oxygenation. Cultures were monitored by T.-A. Chen using monoclonal



antibody/ELISA procedures. Treatments were as follows:

- NJAY-MLO/Air/SerFrac Medium
- NJAY-MLO/Air/M1D Medium
- NJAY-MLO/Air/PL-1M Medium
- NJAY-MLO/Air/PL4-1 Medium
- NJAY-MLO/Air/PL4-2 Medium
- NJAY-MLO/Air/PL4-3 Medium
- NJAY-MLO/Air/PL4-4 Medium
- NJAY-MLO/Air/PL4-5 Medium

### **Pasteuria Cultivation Experiment**

Experiment 24 - Determination of Pasteuria oxygen needs. Media designed for cultivation of MLOs, although unsuccessful for sustained cultivation of these microbes, when amended with components applicable to the "nematode pseudocoelomic microhabitat," were effective for cultivation of another, heretofore uncultivable, group of potentially important microbes, the plant nematode-parasitic actinomycetes, Pasteuria spp. Ironically, since Pasteuria spp. grow very slowly in these media, requiring 4-6 weeks between transfers, they might be excellent co-culture organisms for MLO culture. To begin the process of determining under which culture conditions they might be used, the oxygen requirements of Pasteuria were investigated. The treatments were as follows:

- Pasteuria/Air
- Pasteuria/Anaerobic Hood





## RESULTS AND DISCUSSION

### Comparison of Methods for Determining Growth.

Although we used many techniques (light microscopy, bioassay, gene probes, monoclonal antibody/ELISA) for monitoring MLOs during these studies, bioassay is the only one that measures viability. Problems, however, ensued in using this technique. Unexpectedly, the NJAY-MLO appears to be pathogenic for M. fasciifrons. Also, some media were probably toxic. In general, high mortality often made estimation of viability by disease production unreliable. For this reason, future experimentation should focus on use of gene probes or ELISA to monitor MLO titer by time course studies. Some progress toward this end was achieved.

In collaboration with B. Kirkpatrick at UC-Davis, we were able to observe an increase in gene probe intensity (e.g., Experiment 14: Table 5, 0→3 on intensity rank for PYLR-MLO cultures in DMCMH medium in anerobic hood; Experiment 15: Table 6, 2→5 for PYLR MLO in M1D medium) in dot blots of DNA extracted culture samples. The increase tended to peak at 1-2 days, followed by a slow decline. This was corroborated in experiments with B. Sears at Michigan State University (Experiments 28 & 29: Tables 17 & 18). In these experiments, increases of up to 25-fold were observed. The gene probe results therefore suggest that the MLOs are increasing under the conditions of cultivation, but are unable to sustain growth.

ELISA, at Rutgers and UC-Davis, also indicated a 1-2 day



burst of growth, followed by detection of the antigen for more than a month. For example, in Experiment 14 (Table 5), the optical reading in Chang medium increased from .38→.50 at Day 1. These results, taken together, show promise for both methods in the monitoring of MLO growth.

Some questions remain to be answered. For example, in many experiments, e.g., Experiment 15 (Table 8, Without Yeast, Air, Passage 1, 10°), it is not clear, when starting with large amounts of inoculum (i.e., high optical readings), whether there has been a steady decline in the titer of organisms, or whether some limited growth was obscured by the presence of large amounts of antigen. That some growth did occur is suggested by 10<sup>-1</sup> subculture of these same cultures, which showed an increase in optical reading from Day 0→1→2→3, with a leveling off at Day 6. (It should be noted that during these cultivation attempts, fresh medium is typically added at an amount about 1/5 of the culture volume every 2 days. In the cases discussed here, much larger additions (higher dilutions) were made, with ATP and fresh plant extract as amendments. Maintenance of apparently stable values may therefore indicate growth.)

Although many different types of pleomorphic structures were seen in MLO culture attempts, none could be definitively shown to be MLOs. Therefore, dark-field microscopy is probably an unreliable method for determining the presence of MLOs. This conclusion is not, however, categorical. Pleomorphic, budding structures were present in cultures that tested positive by ELISA





for acholeplasmas and NJAY-MLO in Experiments 23 and 25. More work, particularly electron microscopy, needs to be done to verify whether these structures are MLOs. If they are, dark-field microscopy could be a valuable tool in monitoring MLO titers.

#### Comparison of Cultivation Components.

1. Sources of Inoculum. The sources of inoculum used in these experiments were infected leafhoppers (hemolymph or macerates of whole insects) and diseased plants [celery, aster, lettuce, and evening primrose (*Oenothera* sp.)]. MLO isolates that exhibited an initial burst of growth in the culture systems were obtained from whole leafhopper macerates (Experiments 17-19), celery (Experiments 13-15), and evening primrose (Experiments 28, 29). In Experiment 15, fresh celery extracts, when added to aerobic (but not anaerobic) cultures at Day 2, appeared to result in an increase in PYLR-MLO growth by Day 3 (Table 6). This was particularly true for primary cultures that had been diluted 10-fold (Table 8: Without Yeast, Air, Passage 1,  $10^{-1}$ ). There was some evidence for isolation of MLOs from leafhopper hemolymph as well (Experiment 23, Table 16, slight ELISA reaction of NJAY-MLO pass 4 to NJAY-MLO-specific antisera.). However, the preponderance of evidence suggests that either whole leafhopper macerates, or, particularly, macerates of highly diseased celery, were the best sources of inoculum used in these experiments.



2. Extraction Protocols. Initial survival of MLOs was accomplished using many extraction protocols, including (i) maceration of leafhoppers in air at ambient temperatures (Experiments 3-5, 10, 13, 16), (ii) on ice with refrigerator storage (Experiments 17, 18), or (iii) on ice in a cold room (Experiments 25, 26, 30), (iv) maceration of leafhoppers in an anaerobic hood (Experiments 16, 22), (v) maceration of celery in an anaerobic hood (Experiment 15), and maceration of infected evening primrose leaf tip cultures (Experiments 28, 29). Various extraction media proved efficacious, particularly the highly reducing medium, MEMEK1, designed for these studies (Experiments 17, 18), and various bacterial culture media (Experiments 14-16, 28, 29: Pl-1, PL3 series, P1, Chang, DMCMH, M1D, CBY) and insect tissue culture media (Experiments 3, 4, 10: DCCM, AC-20M). Use of traditional extraction solutions, such as phosphate buffered saline (Experiments 17, 18: Table 13) or sulfite-containing-media (Experiments 6, 7: Table 4) or buffers (Experiments 8, 9: Table 4), with the exception of BEM2 (Experiment 14: Table 5) and, possibly, BEM1 (Experiment 13: Table 10), were not nearly as effective.

3. Culture Conditions. The two principal comparisons made in these studies were between isolation and culture (i) at ambient vs. cool or cold temperatures, in (ii) aerobic vs. anaerobic environments. Two types of anaerobic system were used: GasPaks, in which isolations were accomplished in air, with transferral of





isolates to the GasPaks; anaerobic hoods, in which isolations were usually performed inside the hoods. Two additional means of controlling the amount of oxygen available to the MLO cells were to use a mineral oil layer over the cultures, and to use tubes instead of microtiter wells.

Isolation on ice, with maintenance of cultures in the cold (at 4°C) was clearly superior with regards to AY-MLO survival (Experiments 17-19: Tables 4, 13). Although it is not likely that such frigid conditions would be suitable for MLO culture, this method might nevertheless be a method for maintaining MLOs until toxins (from extracted leafhoppers or plants) dissipate or the MLOs can produce metabolites necessary for their growth. In general, there is every reason to believe that maintenance of cool conditions during isolation is preferable. However, the optimal temperature for culture cannot be deduced from these studies. Increases in MLO titers occurred in cultures incubated from 4°C (Experiments 17, 18: Table 13) to 28°C (Experiments 13-15: Tables 5-7, 10), the complete span of temperatures tested.

Cultures were able to increase in air in tubes at 4°C (Experiments 17, 18: Table 13) or microtiter plates at 28°C (Experiments 13-15: Tables 5-7, 10). As shown in Table 8 (Experiment 15), growth occurred in both aerobic and anaerobic environments. However, there was a striking difference between titers obtained from aerobic vs. anaerobic culture, with, as detected by ELISA, significantly higher titers of PYLR-MLOs in air (Tables 8-9: Day 1), but perhaps less continued maintenance



of MLO titers in air (Tables 8-9: Day 6). Counterintuitively, there was evidence (by gene probe) of a burst of growth in air after addition of fresh celery extract at Day 2 (Table 6). It is generally believed that oxygen would exacerbate damage by plant toxins; however, if the MLOs require oxygen, and plant extracts have nutrients needed for MLO culture, the observed growth is entirely explainable. Certainly, evidence accumulated in later experiments (Experiments 22-23, 25-26) do not support the contention that NJAY-MLO grows better in anaerobic environments. Also, oxygen-excluding mineral oil layers appeared to decrease, rather than increase, NJAY-MLO survival (Experiment 19: Table 4). With possible MLO affinity to the aerobic acholeplasmas (Lim and Sears, 1989), this is not surprising. Perhaps future experiments should be undertaken in systems, such as tissue culture flasks (with or without solid media) that allow easy penetration of oxygen. Aeration of cultures should also be considered.

4. Cultivation Systems. Together with the somewhat enigmatic increase in AY-MLOs at 4°C in MIMIK1 (Experiment 18, Table 13) and PL-1 (Experiment 19, Table 4) media, three cultivation systems especially showed promise: (i) cultivation of Oenothera-MLO, extracted from leaf tip cultures, in PL3 series, PL-1M, and SerFrac media, under aerobic conditions at 28°C (Experiments 28, 29: (ii) cultivation of PYLR-MLO, extracted from celery, in various media (particularly P1, Chang and M1D media), under aerobic conditions at 28°C (Experiments 14, 15);





(iii) cultivation of NJAY-MLO, extracted from leafhoppers, in various media (particularly PL-1, SerFrac, and SerFree0), under aerobic conditions at 26°C (Experiments 22-23, 25-26). Evidence for the success of the first two systems was obtained through time course monitoring of cultures by gene probe and ELISA; evidence for the success of the latter systems was obtained through microscopic and ELISA monitoring of cultures for long time periods, over many passages.

These methods suggest several conclusions:

1. All three monitoring systems (bioassay, gene probe, ELISA) used in these studies were adequate, but provided different clues (viability, genome multiplication, and cell growth, respectively) as to the progress of the cultures. Light microscopy may yet be found to have application, but much work needs to be done in this regard with confirmation of cultured organisms by electron microscopy.

2. Cultivation of several, somewhat unrelated, MLOs may be achievable (including, *Oenothera* and PYLR-MLOs, BLTVA, and aster yellows MLOs from New Jersey, Canada, and the western United States). Note: the taxonomic identities and phylogenetic relationships of these MLOs have not been well established; some, of these MLOs may be in the same taxon. Even MLO strain differences may prove to be important in cultivation success.

3. Whole leafhoppers (or possibly their hemolymph) or diseased celery may be suitable sources of inoculum for cultivation trials. However, the best source used in these



experiments may have been leaf tip cultures.

4. While extraction should be performed on ice, preferably in a cold room, cultivation at 28°C is probably satisfactory. Whether cultivation at 26°C or 30°C would be better cannot be ascertained from these studies. However, poor survival of AY-MLO at temperatures above 32°C suggest that higher temperatures should be avoided.

5. Efforts should be made to increase oxygenation in culture systems. This does not, however, rule out the possibility that extractions might be less harmful to MLOs if carried out under totally anaerobic conditions. Perhaps, after a period of adaptation under anaerobiosis or refrigeration in which toxins are metabolized or inactivated or essential metabolites are allowed to accumulate under anaerobiosis, cultures could be gradually weaned to oxygen-containing atmospheres and higher temperatures.

6. Media shown to be useful for cultivation of MLOs (i) were based on components found in phloem saps (P1, PL-1, PL-1M, PL-3), (ii) had plant extract additives (Chang, or fresh celery extract), (iii) were based on a knowledge of insect hemolymph (M1D), or (iv) were media proven to be successful for cultivation of achleoplasmas (SerFrac, SerFree). A successful strategy might be one in which a medium with a large number of chemically defined components (e.g., P1, PL-1, PL3-2: Tables 18, 22A) is supplemented with plant extracts and other complex nutrient sources (such as has been done in the past in formulation of



Chang and M1D media, respectively, and in the PL3 series media here).

7. Components (as summarized in Appendix 1) found to be important in obtaining initial bursts of MLO growth in culture appeared to be ones involved with membrane stability, e.g., mOsm > 500, and high concentrations of choline chloride, BSA, polyamines, and glycerol in the media. High concentrations of amino acids (> 16 g/l) also appeared to encourage growth. Serum, yeast extract, and high concentrations of tryptone appeared to be detrimental.

8. None of the co-culture systems (Experiments 1-4, 8, 15, 20-21, 27: DU-E beetle cells, AC-20 leafhopper cells, yeast, bacteria) appeared to improve survival of MLOs. The yeast, as utilized here, may have been detrimental (Experiment 15: Tables 6, 8). However, the striking success in use of these systems for cultivation of other fastidious mollicutes (including spiroplasmas and mycoplasmas), suggests that this line of research should not be abandoned.





## Studies on the Biology of the New Jersey Aster Yellows Agent

Some features of the vector relationship of the NJAY MLO were determined. After a 4 day acquisition access by 1st instar Macrosteles fascifrons nymphs, followed by a latent period of 14-25 days (mean = 17.9), 59-66% of surviving adult leafhoppers transmitted the pathogen for 9-46 days; maximum transmission occurred from 25 to 39 days after the acquisition access period. Plant symptoms appeared 2-4 weeks after infected leafhoppers were placed on young healthy plants. Symptoms were characteristic of aster yellows, and included floral virescence. In contrast to classical strains of AY, however, NJAY-MLO appeared (Fig. 1) to be pathogenic to M. fascifrons ( $p < 0.05$  in Mann-Whitney and Wilcoxon Ranked Sum tests; infected  $n = 23$ , noninfected  $n = 12$ ). Similar survivorships were observed for males and females. Also, the two sexes appeared to transmit the disease agent at similar rates: 33.3% (female), and 23.7% (male). Observation of pathogenicity for the vector suggests that, unlike many strains of AY agents in the eastern U. S. transmitted principally by M. fascifrons, NJAY may be transmitted by another vector in the field. The failure of NJAY-MLO monoclonal antibodies to react with other AY strains may, therefore, reflect basic biological differences in field maintenance.



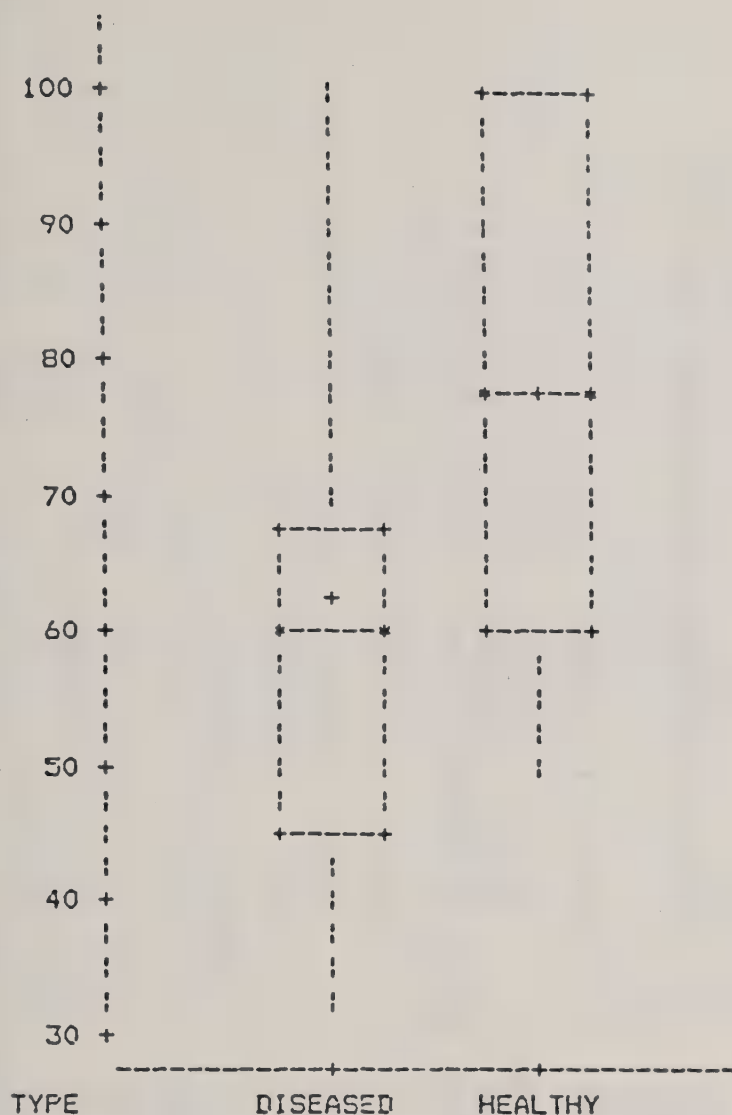


Fig. 3. Schematic plot of longevities of healthy Macrosteles fascifrons leafhoppers, and of leafhoppers that transmitted NJAY MLOs to aster. Ordinate = longevity in days. The mean longevities of the two classes of leafhoppers were significantly different statistically (see text).





Table 4. Cultivation Trials

Inoculum			Extraction Procedure		Culture System			Assay Results				
Type	Source	Type	Medium	Temp/Atm	Medium	Cells	Vessel	Temp/Atm	Type	Day	%Symp	ELISA Probe
1 NJAY	M. f.	1	DCCM	Amb/Air	DCCM	DU-E	24-well	26°C/Air	Bio	0-7	0	
						DU-E/LD-1					0	
2 NJAY	M. f.	1	DCCM	Amb/Air	DCCM	DU-E	24-well	26°C/Air	Bio	0-4	0	
3 NJAY	M. f.	1	DCCM	Amb/Air	DCCM	DU-E	24-well	26°C/Air	Bio	0	18%	
										2-7	0	
4 NJAY	M. f.	1	AC20M	Amb/Air	AC20M	AC-20	24-well	26°C/Air	Bio	0	33%	
						& yeast in insert						
5 NJAY	M. f.	1	M1D	Amb/Air	M1D		Tube	Amb/Air	Bio	2-4	0	
6 NJAY	Ast.-Pet. Ast.-Lam.	1	M1D-SO <sub>3</sub>	Ice/Air	M1D		Tube	Amb/Air	Bio	0	4%	
7 NJAY	Ast.-Pet. (petioles were shell frozen before extraction)	1	M1D-SO <sub>3</sub>	Ice/Air	M1D		Tube	Amb/Air	Bio	0	0	
8 NJAY	M. f.	2	Gly-Mg	Ice/Air	AC20M	AC-20	24-well	26°C/Air	Bio	0-2	0	
9 NJAY	Lettuce	3	Percoll	Amb/Air	C-3G		Tube	Amb/Air	Bio	0	0 <sup>M</sup>	
10 NJAY	M. f.	1	AC20M	Amb/Air	AC20M		Tube	Amb/Air	Bio F	0	33%	
									Bio M	0	24%	
11 S. a.	Culture				DCCM	DU-E	24-well	26°C/Air	Microscope	(see Fig. 2, p. 39)		
					DCCM	Yeast						
					AC20M	AC-20						
12 S. k.	Culture				DCCM	DU-E	24-well	26°C/Air	Microscope	(see Fig. 3 p. 40)		
					DCCM	Yeast						
					AC20M	AC-20						



xpt Inoculum			Extraction Procedure			Culture System			Assay Results		
Type	Source	Type	Medium	Temp/Atm	Medium	Cells	Vessel	Temp/Atm	Type	Day	%Symp ELISA Probe
3	BLTVA	Leafhp	4 BEM1	Ice/Air	Chang		24-well	28°C/Air GasPak	ELISA	(see Tables 7, 9)	
	SAY	Celery	5 BEM1	Ice/Air	Chang		24-well	28°C/Air GasPak			
4	BLTVA	Celery	5 BEM2	Ice/Air	Chang DMCMH		24-well	28°C/Air GasPak	ELISA	& Gene Probe (see Tables 2, 7)	
					Chang DMCMH			28°C/Hood1			
	PYLR	Celery	5 BEM2	Ice/Air	Chang DMCMH			28°C/Air			
					Chang DMCMH			28°C/Hood1			
5	PYLR	Celery	5 M1D	28C/Hood1	M1D		24-well	28°C/Air 28°C/Hood1 28°C/Air 28°C/Hood1	ELISA	& Gene Probe (see Tables 3, 5-6)	
						Yeast		28°C/Hood1 28°C/Air 28°C/Hood1			
			Chang	28C/Hood1	Chang		24-well	28°C/Air 28°C/Hood1 28°C/Air 28°C/Hood1			
						Yeast		28°C/Hood1 28°C/Air 28°C/Hood1			
			CBY	28C/Hood1	CBY		24-well	28°C/Air 28°C/Hood1 28°C/Air 28°C/Hood1			
						Yeast		28°C/Hood1 28°C/Air 28°C/Hood1 28°C/Air 28°C/Hood1			
			P1	28C/Hood1	P1		24-well	28°C/Air 28°C/Hood1 28°C/Air 28°C/Hood1			
						Yeast		28°C/Hood1 28°C/Air 28°C/Hood1 28°C/Air 28°C/Hood1			
6	PYLR	Leafhp	4 M1D Chang CBY P1	28C/Hood1	M1D Chang CBY P1		24-well	28°C/Hood1 28°C/Air 28°C/Hood1 28°C/Air 28°C/Hood1	ELISA	& Gene Probe (see Tables 4, 8)	



xpt Inoculum			Extraction Procedure		Culture System		Assay Results					
Type	Source	Type	Medium	Temp/Atm	Medium	Cells	Vessel	Temp/Atm	Type	Day	%Symp	ELISA Probe
7	EAY	M. f.	6	MEMEK1 PBS	Amb/Air	MIMEK1 PBS	Tube	4°C/Air	Bio	(0-5 Days)	Titer 16→42% 0%	
8	WAY	M. f.	6	MEMEK1 PBS	Amb/Air	MIMEK1 PBS	Tube	4°C/Air	Bio	(0-4 Days)	Titer 20→50% 0%	
9	NJAY	M. f.	1	PL-1	Ice/Air	PL-1	24-well	26°C/Air	Bio	(see Table 10)	0%	
								4°C/Air			0%	
							24-well	26°C/Oil	Bio		0%	
								4°C/Oil			0%	
0	NJAY	M. f.	1	PL-1	Ice/Air	Luria Blood Milk PL-1 Luria Blood Milk PL-1 Luria	Bact1 Solid Solid Solid Liquid Solid Solid Solid Liquid Solid	26°C/Air 26°C/Air 26°C/Air 26°C/Air 26°C/Air 26°C/Air 26°C/Air 26°C/Air 26°C/Air	Micr Micr Micr Micr Micr Micr Micr Micr Micr	0-9 0-9 0-9 0-9 0-9 0-9 0-9 0-9 0-9	No No No No No No No No No	MLO Colonies MLO Colonies MLO Colonies MLO Colonies MLO Colonies MLO Colonies MLO Colonies MLO Colonies MLO Colonies
1	NJAY	M. f.	1	PL-1	Ice/Air	Luria	BacMx	Amb/Hood2	Bio	0-4	0%	(Table 13)
2	NJAY	M. f.	1	PL-2	Amb/Hood2	PL-2A	Solid	26°C/Air	Bio	0-4	0%	(Table 13)
3	NJAY	M. f.	7	PL-2	Amb/Air	PL-2A	Solid	26°C/Air	Bio	0-4	0%	(Table 13)
							Tube	Amb/Hood2	Bio	0-4	0%	(Table 13)
4	Pastr	Culture				R-1	24-well	Amb/Hood2	Micr	14	Moderate Growth	Moderate Growth









xpt Inoculum		Extraction Procedure		Culture System		Assay Results							
Type	Source	Type	Medium	Temp/Atm	Medium	Cells	Vessel	Temp/Atm	Type	Day	%Symp	ELISA	Probe
												*	(X)**
8	Oeno	Explant	8	BM6	Ice/Air	SerFrac	Tube	28°C/Air	Gene Probe (see Table 14)				4
9	Oeno	Explant	8	PL3-2	Ice/Air	SerFrac	Tube	28°C/Air	Gene Probe (Sears)				ND
						PL3-1			Gene Probe (see Table 15)				15
						PL3-2							15
						PL3-3							15
						PL3-4							20
						PL3-5							20
						PL3-1M							25
						PL3-2S							20
						PL3-2Y							15
						PL3-2SY							20
						PL3-P							10
10	NJAY	Leafhp	6	SerFrac	Cold/Air	SerFrac	MicTb	28°C/Air	ELISA			+	18
				M1D		M1D			(see Table 16)			-	
				PL-1M		PL-1M						-	
				PL4-1		PL4-1						-	
				PL4-2		PL4-2						-	
				PL4-3		PL4-3						-	
				PL4-4		PL4-4						-	
				PL4-5		PL4-5						-	

\* Any increase in ELISA reading is noted by +.

\*\* Data are magnitude of increase at day 3 to 14, with average initial blot intensity of about 1 (for Experiment 29, Trial 2, supercoiled DNA was used).





Legend for Table 1.

24-well = Twenty four well microtiter plate  
AC-20 = AC-20 leafhopper cell line  
Amb = ambient (24-28°C)  
Ast.-Lam. = aster laminae  
Ast.-Pet. = aster petioles  
Atm = atmosphere  
Bact1 = small motile bacillus associated with leafhoppers  
BacMx = mixture of bacteria associated with leafhoppers  
Bio = bioassay  
BLTVA = beet leafhopper transmitted virescence agent  
ELISA = enzyme linked immunosorbent assay  
Cold = extraction was done in cold room on ice  
DU-E = Diabrotica undecimpunctata cell line  
EAY = eastern strain of aster yellows  
Explant = plant leaf tip tissue cultures  
Expt = experiment  
F= female  
GasPak = anaerobic jar containing 95% CO<sub>2</sub>, 5% H<sub>2</sub>  
Hood1 = anaerobic hood containing 90% N<sub>2</sub>, 10% H<sub>2</sub>  
Hood2 = anaerobic hood containing 90% N<sub>2</sub>, 5% CO<sub>2</sub>, 5% H<sub>2</sub>  
Ice = extraction was performed on ice  
Leafhp = leafhoppers  
<sup>M</sup> = high mortality of test insects  
M = male  
M. f. = Macrosteles fascifrons  
Micr = observation and enumeration by dark field microscopy  
MicTb = microfuge tube  
NJAY = New Jersey strain of aster yellows MLO  
Oeno = Oenothera-MLO infected leafhoppers  
Pastr = Pasteuria penetrans (a nematode pathogen)  
PYLR = potato yellow leaf roll strain of Western-X MLO  
Probe = gene probe  
SAY = severe strain of western aster yellows MLO  
SFC-SFRO = serum fraction and serum free (no Tween 80) MLO cultures  
Solid = solid agar plate  
Symp% = % plants that developed aster yellows disease after  
          inoculated leafhoppers had fed on them  
Temp = temperature  
Tex2 = hymenopteran parasite cell line  
Tube = 12 x 75 mm culture tube  
Yeast = yeast supplied by D. L. Williamson  
Also, see: List of Media; Extraction Procedures



Table 5. Growth of PYLR in Chang and DMCMH Media, as Determined by Polyclonal Antibody/ELISA & [Gene Probe ranked by intensity of dot on X-ray film] (Experiment 14)

	Day 0	Day 1	Day 2	Day 17
Culture in Chang Medium				
Air	.38	.50	.50	
GasPak	.38	.48	.51	[2]*
Culture in DMCMH Medium				
Air	.29	.30	.32	
GasPak	.29	.31	.31	[1]*
-> Anaerobic Hood			[3]**	
Chang Medium Control				
Air			.01	
GasPak			.01	
DMCMH Medium Control				
Air			.01	
PYLR Celery Control	1.21			
Healthy Celery Control	.03			
[Gene Probe Control]				[0]

\* = Pass 2, 1:10

\*\* = Pass 3, 1:10; transferred to Anaerobic Hood on Day 7



Table 6. Growth of PYLR in Chang, M1D, CBY, and P1 Media, as

Determined by Reaction with an rRNA Gene Probe to PYLR  
(ranked by intensity of dot on X-ray film) (Expt. 15)

	Day 0	Day 1	Day 2	Day 3	Day 6
<u>With Yeast Co-Culture</u>					
Culture in Chang Medium					
Air	1	1	0	0	0
Anaerobic	1	1	1	1	1
Culture in M1D Medium					
Air	1	3	1	2	1
Anaerobic	1	2	2	1	1
Culture in CBY Medium					
Air	1	0	0	0	0
Anaerobic	1	0	0	0	0
Culture in P1 Medium					
Air	3	3	1	2	2
Anaerobic	3	4	3	2	0
<u>Without Yeast Co-Culture</u>					
Culture in Chang Medium		ATP	FPE	ATP	
Air	4	4	1 ↓	2 ↓	0
Anaerobic	5	4	3 ↓	1 ↓	2
Culture in M1D Medium					
Air	2	5	1 ↓	7 ↓	0
Anaerobic	?	4	3 ↓	1 ↓	2
Culture in CBY Medium					
Air	1	1 ↓	1 ↓	3 ↓	0
Anaerobic	4	1 ↓	1 ↓	2 ↓	ND
Culture in P1 Medium					
Air	8	7 ↓	1 ↓	2 ↓	0
Anaerobic	10	5 ↓	3 ↓	3 ↓	ND
<u>Media Controls</u>					
With Yeast					
Air					
M1D		0			0
Anaerobic					
M1D		0			0
P1		0			0
Without Yeast					
Air					
M1D		0			0
P1		0			0
<u>Healthy Plant Extract Controls</u>					
In M1D Medium		0			0
In P1 Medium		0			?

ND = not determined;

↓ = ATP or Fresh plant extract added to culture





Table 7. Detection/Growth of PYLR, Extracted From Leafhoppers, In Anaerobic Hood, as Determined by Reaction with an rRNA Gene Probe to PYLR (ranked by intensity of dot on X-ray film) (Experiment 16)

	Day 0	Day 7
Chang Medium		
PYLR Diseased Celery	1	
Healthy Celery Control	0	
M1D Medium		
PYRL Diseased Celery	1	
CBY Medium		
Healthy Celery Control	0	
P1 Medium		
PYLR Diseased Celery	1	0
Healthy Celery Control	0	

Film (Growth of Film) Exposed  
 Determined by Reaction with an RPN (see Table)  
 (Intensity of dot on X-ray film) (Experiment 18)

Day 0

and Celery	
for Control	0
	1
for Control	0
	1
and Celery	
Control	0

Table 8. Growth of PYLR in Chang, M1D, CBY, and P1 Media, as Determined by Reaction with Polyclonal Antibody/ELISA (Experiment 15) [aveages of two values]

	Day 0	Day 1	Day 2	Day 3	Day 6
<u>With Yeast</u>					
Air					
Passage 1 ( $10^{-1}$ )					
Chang Medium	.143	.208*	.280*	.263*	.177
M1D Medium	.202	.115	.443*	.076	.013
CBY Medium	.137	.184	.278*	.280*	.279*
P1 Medium .140	.207	.154	.168	.136	
Anaerobic Hood					
Passage 1 ( $10^{-1}$ )					
Chang Medium	.164	.226	.276	.250	
M1D Medium	.143	.063	.051	.050	
CBY Medium	.112	.169	.156	.166	
P1 Medium	.140	.174	.158	.168	
<u>Without Yeast</u>					
Air					
Passage 1 ( $10^0$ )					
Chang Medium	.798	.731	ATP .719	FPE ↓ .508(.45)	ATP ↓.419
M1D Medium	.686	.635	.607	↓ .454(.37)	↓.207
CBY Medium	.583	.540	↓ .516	↓ .376(.32)	↓.322
P1 Medium	.615	.650	↓ .603	↓ .510(.29)	↓.422
Passage 1 ( $10^{-1}$ )					
Chang Medium	.124	.124	.138	↓ .152	↓.149
M1D Medium	.117	.136	.142	↓ .128	↓.091
CBY Medium	.104	.101	↓ .105	↓ .121	↓.125
P1 Medium	.132	.187	↓ .182	↓ .185	↓.174
Anaerobic Hood					
Passage 1 ( $10^0$ )					
Chang Medium	.798	.677	.694	↓ .533(.43)	↓.504
M1D Medium	.686	.558	.563	↓ .446(.35)	↓.437
CBY Medium	.583	.469	↓ .465	↓ .359(.29)	↓.336
P1 Medium	.615	.503	↓ .479	↓ .419(.30)	↓.410
Passage 1 ( $10^{-1}$ )					
Chang Medium	.135(.15)	.161	.180	↓ .194	↓.217
M1D Medium	.156(.14)	.123	.137	↓ .118	↓.125
CBY Medium	.075(.12)	.113	↓ .113	↓ .113	↓.121
P1 Medium	.112(.12)	.146	↓ .142	↓ .102	↓.042
Passage 2 ( $10^{-1}$ )					
Chang Medium	.339	.344		.365	
M1D Medium	.319	.293		.277	
CBY Medium	.231	.218		.205	
P1 Medium	.272	.254		.248	

\* = precipitate in well, from yeast growth, may have interfered with measurements; ↓ = ATP, or Fresh plant extract added





Table 9. Comparison of PYLR Titers in Air and Anerobic Hood Without Yeast, Passage 1, as Determined by Reaction with Polyclonal Antibody/ELISA (Experiment 15)

	<u>Air</u>	<u>Anaerobic Hood</u>
DAY 1		
Chang Medium	.731	.677
M1D Medium	.635	.558
CBY Medium	.540	.469
P1 Medium	.650	.503
DAY 6		
Chang Medium	.419	.504
M1D Medium	.207	.437
CBY Medium	.322	.336
P1 Medium	.422	.410



Table 10. Growth Titers of Selected PYLR (Experiment 14), BLTVA (Experiment 13), and SAY (Experiment 13) Cultures in Chang And DCMCH Media, as Determined by Reaction with Polyclonal and Monoclonal Antibody/ELISA

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POLYCLONAL Ab

Celery Inoculum

SAY (Pass 2, Chang Medium)	.077
PYLR	
(Pass 1, Chang Medium, GasPak)	.394
(Pass 2, DMCMH Medium, Air)	.106
PYRL Celery Control	1.251

Leafhopper Inoculum

BLTVA (Pass 2, Chang Medium)	.069
BLTVA Celery Control	.071

MONOCLONAL Ab

Celery Inoculum

SAY (Pass 2, Chang Medium)	.065
PYLR	
(Pass 1, Chang Medium, GasPak)	.344
(Pass 2, DMCMH Medium, Air)	.113
PYRL Celery Control	1.314

Leafhopper Inoculum

BLTVA (Pass 2, Chang Medium)	.068
BLTVA Celery Control	.057

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Table 11. Detection/Growth of PYLR, Extracted From Leafhoppers, In Anaerobic Hood, as Determined by Reaction with Polyclonal and Monoclonal Antibody/ELISA (Expt. 16)

	Day 0	Day 1	Day 4	Day 6
Chang Medium				
PYRL ( $10^{-1}$ )	.050	.045	.039	
Healthy LH Control	.034	.038	.036	
Medium Control (Anaer.)				.026
M1D Medium				
PYRL ( $10^{-1}$ )	.036	.034	.033	
Healthy LH Control	.023	.028	.025	
Medium Control (Air)				.016
Medium Control (Anaer.)				.021
CBY Medium				
PYRL ( $10^{-1}$ )	.041	.034	.032	
Healthy LH Control	ND	ND	.013	
Medium Control (Air)				.017
Medium Control (Anaer.)				.021
P1 Medium				
PYRL ( $10^{-1}$ )	.028	.025	.025	
Healthy LH Control	.024	.026	.024	
Medium Control (Air)				.016
Medium Control (Anaer.)				.017

ND - not determined; LH = leafhopper; Anaer. = anaerobic hood





Table 12. Growth Titers of BLTVA, SAY, and PYLR in Chang and DMCMH Media, as Determined by Reaction with Polyclonal and Monoclonal Antibody/ELISA (Expt. 13 & 14)

	Day 9	Day 15
Healthy Leafhopper Control (ave. of 4)		.024
Media Control (ave. of 4)		.020
BLTVA		
Pass 2, Air, Chang Medium		.026
DMCMH Medium		.017
Pass 2, GasPak, Chang Medium		.024
SAY		
Pass 2, Air, Chang Medium		.024
Pass 2, GasPak, Chang Medium		.029
Anaerobic Hood, Chang Medium	.031	
PYLR		
Pass 2, GasPak, Chang Medium		.086
DMCMH Medium		.108
Anaerobic Hood, Chang Medium	.347	



Table 13. Maintenance of AY-MLO in MIMEK1 cf. PBS, at 4°C, in Air, as Determined by % Disease Transmission of Injected Leafhoppers (Experiments 17 & 18)

	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
SAY-MLO						
MIMEK1 Medium	16	42	10			0
PBS	3	0	0			0
WAY-MLO						
MIMEK1 Medium	20	4	0	38	50	
PBS	47	0	0	0		



Table 14. Growth of NJAY-MLO in PL-1, M1D, SerFree, and SerFrac Media, as Determined by Reaction in ELISA with a Mixture of 6 NJAY-MLO-specific Monoclonal Antibodies and Acholeplasma-specific Polyclonal Antibodies (Experiment 25)

	<u>Antibody</u>			
	<u>NJAY</u>	<u>A.ax</u>	<u>A.gr</u>	<u>A.ld</u>
CONTROLS				
PBS Control	.000	.000	.000	.000
SerFree0 Medium Control	.025	.073	.062	.000
SerFrac Medium Control	.000	.005	.004	.284*
Healthy Lettuce Control		.061	.122	.104
NJAY-MLO Control	>2	.062	.051	.103

Also, Healthy Rabbit Serum reacted with NJAY-MLO at 0.084, with Healthy Lettuce at .055.

#### AIR

SerFree0, Pass 1, 10-1	.330	.167	.022	.063
10-2	.083	.211	.000	.071
10-3	.000	.152	.000	.047
SerFree1, Pass 1, 10-2	.022	.000	.000	.000
SerFree2, Pass 1, 10-2	.000	.000	.000	.000
SerFree4, Pass 1, 10-1	.073	.055	.151	.063
SerFrac, Pass 1, 10-1	.287	.173	.008	.238
10-2	.054	.187	.000	.219
10-3	.057	.148	.000	.414

#### ANAEROBIC HOOD

SerFree0, Pass 1, 10-2	.087	.047	.450	.309
SerFree1, Pass 1, 10-2	.000	.000	.003	.000
SerFree2, Pass 1, 10-2	.055	.000	.009	.071
SerFree4, Pass 1, 10-2	.000	.051	.186	.155
Pass 2, 10-1	.026	.019	.051	.017
SerFrac, Pass 1, 10-2	.028	.000	.006	.344

A.ax = Acholeplasma axanthum; A.gr = A. granularum;

A.ld = A. laidlawii





Table 15. Growth of NJAY-MLO in PL-1, M1D, SerFree, and SerFrac Media, as Determined by Reaction in ELISA with a Mixture of 6 NJAY-MLO-specific Monoclonal Antibodies and Acholeplasma-specific Polyclonal Antibodies (Experiment 26)

	<u>Antibody</u>			
	<u>NJAY</u>	<u>A.ax</u>	<u>A.gr</u>	<u>A.ld</u>
CONTROLS				
PBS Control	.000	.000	.000	.000
SerFree1 Medium Control	.000	.000	.005	.453*
M1D Medium Control	.000	.000	.002	.256*
PL-1 Medium Control	.000	.000	.000	.143*
Healthy Lettuce Control		.061	.122	.104
NJAY-MLO Control	>2	.062	.051	.103
Also, Healthy Rabbit Serum reacted with NJAY-MLO at 0.084, with Healthy Lettuce at .055.				
AIR				
SerFree1, Pass 1, 10-2	.015	.060	.020	.313
SerFree4, Pass 1, 10-2	.000	.000	.008	.418
M1D, Pass 1, 10-2	.000	.000	.049	.191
Pass 2, 10-1	.002	.000	.000	.164
PL-1, Pass 1, 10-1	.071	.000	.000	.347
10-2	.055	.000	.000	.264
ANAEROBIC HOOD				
SerFree1, Pass 1, 10-2	.050	.000	.000	.350
SerFree4, Pass 1, 10-2	.007	.000	.000	.350
M1D, Pass 1, 10-2	.019	.201	.000	.121
PL-1, Pass 1, 10-1	.038	.094	.007	.268
10-2	.048	.006	.000	.385



Table 16. Growth of NJAY-MLO in PL-1 Medium, as Determined by Reaction in ELISA with a Mixture of 6 NJAY-MLO-specific Monoclonal Antibodies and Acholeplasma-specific Polyclonal Antibodies (Experiments 22 & 23)

	<u>Antibody</u>			
	<u>NJAY</u>	<u>A.ax</u>	<u>A.gr</u>	<u>A.ld</u>
CONTROLS				
PBS Control	.000	.000	.000	.000
PL-1 Medium Control	.000	.000	.000	.143*
Healthy Lettuce Control		.061	.122	.104
NJAY-MLO Control	>2	.062	.051	.103
Also, Healthy Rabbit Serum reacted with NJAY-MLO at 0.084, with Healthy Lettuce at .055.				
AIR				
<u>Experiment 22</u> : From agar ---> liquid medium at pass 2.				
PL-1, Pass 4, 10-2	.018	.001	.264	.489
Pass 5, 10-1	.021	.009	.000	.007
<u>Experiment 23</u> : From agar ---> liquid medium at pass 2.				
PL-1, Pass 3, 10-2	.000	.013	.000	.424
Pass 4, 10-1	.012	.026	.000	.308



Table 17. Growth of *Oenothera*-MLO in PL-1, SerFrac and Sears' Broth and Semisolid Media, as Determined by Intensity of Autoradiogram Blots with B. Sears' 4.2 kbp *Oenothera*-MLO Plasmid Probe pmp9 (Experiment 28)

	Passage 1				Passage 2
	Day 0	Day 1	Day 14	Day 28	Day 7
Inoculum	1				
SerFrac		4		4	0.5
PL-1	bad blot	----->			
Sears' Media					
Broth		5		4	0.5
Semisolid		2		2	1





Table 18. Growth of *Oenothera*-MLO in PL3 Series, PL-1M, and SerFrac Media, as Determined by Intensity of Autoradiogram Blots with B. Sears' 4.2 kbp *Oenothera*-MLO Plasmid Probe pmp9 (Experiment 29)

	Day 0	Day 3	Day 11	Day 14
<u>Trial 1</u>				
<b>Supercoiled DNA</b>				
PL3-1	ND	ND	ND	
PL3-2	4	3	1	
PL3-3	1.5	2	1	
PL3-4	3	2	3	
PL3-5	4	3	2.5	
PL-1M	ND	4	3	
PL3-2S	3.5	3	2	
PL3-2Y	3	2.5	2	
PL3-P	2	2	2.5	
PL3-2SY	2	1	1	
<u>Trial 2</u>				
<b>Supercoiled DNA</b>				
PL3-1	ND	ND		15
PL3-2	1	10		15
PL3-3	1	20		15
PL3-4	10	20		15
PL3-5	ND	25		10
PL-1M	10	20		20
PL3-2S	3	15		10
PL3-2Y	2	20		15
PL3-P	1	18		10
PL3-2SY	1	10		8
SerFrac	ND	15		10
<b>Linear DNA</b>				
PL3-1	ND	ND		10
PL3-2	1	1		10
PL3-3	1	3		15
PL3-4	1	3		5
PL3-5	ND	5		10
PL-1M	1	3		3
PL3-2S	1	3		10
PL3-2Y	2.5	3		15
PL3-P	1.5	3		5
PL3-2SY	1	1		5
SerFrac	ND	3		3



Table 19. Growth of NJAY-MLO in PL-4 Series, PL-1M, M1D, and SerFrac Media, as Determined by Reaction in ELISA with a Mixture of 6 NJAY-MLO-specific Monoclonal Antibodies (Experiment 30)

	Day 0		Day3		Day 7	
	10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-1</sup>	10 <sup>-2</sup>
CONTROLS						
NJAY Lettuce	2.0+					
Healthy Lettuce	0.082					
Healthy Leafhoppers in:						
M1D	-0.016		-0.047		-0.098	
NJAY Leafhoppers in:						
M1D	0.006		-0.014		-0.106	
SerFrac	0.171	-0.047	-0.080	0.031	-0.020	0.035
PL-1M	-0.027		-0.081		-0.146	
PL4-1	-0.060		-0.044		-0.105	
PL4-2	-0.062		-0.040		-0.102	
PL4-3	-0.027		-0.091		-0.129	
PL4-4	-0.071		-0.065		0.012	
PL4-5	-0.101		-0.111		0.006	



Table 19. MLO Cultivation Media.			
Component	Schneiders Drosophila Medium	CMRL Media-1066	Media 199
<u>Inorganic salts and buffers (mg/liter)</u>			
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	600 Anh	200 Anh	200 Anh
$\text{CoCl}_2 \cdot 2\text{H}_2\text{O}$			
$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$			
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$			
$\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$			0.72
$\text{H}_3\text{BO}_4$			
Hepes			
KCl	1,600	400	400
$\text{KH}_2\text{PO}_4$			
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$			
MgO			
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	3,700	200	200
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$			
$\text{MnSO}_4$			
NaCl	2,100	6,800	6,800
$\text{NaHCO}_3$	400	2,200	2,200
$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$	1,321		
$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$		140	140
$\text{Na}_2\text{MoO}_4$			
$(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$			
$\text{ZnCl}_2 \cdot 4\text{H}_2\text{O}$			





<u>Amino Acids</u> (mg/liter)			
L-Alanine		25	50
$\beta$ -Alanine	500		
L-Arginine·HCl	400	70	70
L-Asparagine			
L-Aspartic acid	400	30	60
L-Cysteine·HCl	60	260	0.11
L-Cystine·2HCl	100	20	20
L-Glutamic acid	800	75	150
L-Glutamine	1,800	100	100
Glycine	250	50	50
L-Histidine	400	20	22
L-Hydroxyproline		10	10
L-Isoleucine	150	20	40
L-Leucine	150	60	120
L-Lysine·HCl	1,650	70	70
L-Methionine	800	15	30
L-Phenylalanine	150	25	50
L-Proline	1,700	40	40
L-Serine	250	25	50
L-Threonine	350	30	60
L-Tryptophan	100	10	20
L-Tyrosine	500	40	40
L-Valine	300	25	50



Nucleic acid precursors (mg/liter)			
DNA, herring sperm			
RNA, torula yeast			
Adenine			10
Adenosine			
Adenosine monophosphate			0.2
Adenosine triphosphate			1.0
Cytidine			
Cytidine monophosphate			
Cytidine triphosphate			
Guanine			0.3
Guanosine			
Guanosine monophosphate			
Guanosine triphosphate			
Hypoxanthine			0.3
Inosine			
Inosine monophosphate			
Thymidine		10	
Thymidine monophosphate			
Thymidine triphosphate			
Thymine			0.3
Uracil			0.3
Uridine			
Uridine monophosphate			
Uridine 5'-triphosphate		1.0	
Xanthine			0.3
Xanthine monophosphate			
2'-Deoxyadenosine		10	
2'-Deoxyadenosine monophosphate			
2'-Deoxycytidine		10	



2'-Deoxycytidine monophosphate			
2'-Deoxyguanosine		10	
2'-Deoxyguanosine monophosphate			
2'-Deoxyuridine monophosphate			
5-Methyldeoxycytidine		0.1	
Phosphoribosyl pyrophosphate			
D-(-)-Ribose			0.5
Ribose-1-phosphate			
2'-Deoxy-D-ribose			0.5





<u>Cofactors (mg/liter)</u>			
Coccarboxylase		1.0	
Coenzyme A		2.5	
Flavin adenine dinucleotide		1.0	
Nicotinamide adenine dinucleotide		7.0	
Nicotinamide adenine dinucleotide phosphate		1.0	
<u>Vitamins, Reducing agents (mg/liter)</u>			
Ascorbic acid			0.5
p-Aminobenzoic acid			0.05
d-Biotin			0.01
Calciferol			0.1
Calcium pantothenate			0.01
Choline chloride			0.5
Folic acid			0.01
Glutathione (reduced)		10	0.05
i-Inositol			0.05
Menadione			0.01
Niacin (Nicotinic acid)			0.025
Niacinamide			0.025
Pyridoxal·HCl			0.025
Pyridoxal-5-phosphate			
Pyridoxine·HCl			0.025
Riboflavin			0.01
Thiamine·HCl			0.01
α-Tocopherol phosphate			0.01
Vitamin A			0.14
Vitamin B <sub>12</sub>			



Carbohydrates, Sugars, Sugar alcohols, Phosphorylated Sugars, Osmoregulators (mg/liter)

Choline chloride			
Erythrose			
Fructose			
Fructose-6-phosphate			
Glucose	2,000	1,000	1,000
Glucose-6-phosphate			
Glycerol-3-phosphate			
i-Inositol			
Maltose			
Mannitol			
Sodium glucuronate		4.2	
Sorbitol			
Sucrose			
Trehalose	2,000		
Xylose			
<u>Organic acids (mg/liter)</u>			
Acetic acid		83	50
Fumaric acid	100		
$\alpha$ -Ketoglutaric acid	200		
Malic acid	100		
Mevalonic acid			
Oxalacetic acid			
Pyruvic acid			
Shikimic acid			
Succinic acid	100		



<u>Lipids</u> (mg/liter)			
Campesterol			
Cholesterol			0.2
Sitosterol			
Stigmasterol			
Linoleic acid			
Oleic acid			
Palmitic acid			
Glycerol			
DL- $\alpha$ - Glycerophosphate $\cdot 6H_2O$			
Phosphatidic acid			
Phosphatidylcholine			
Phosphatidylcholine, di-oleoyl			
Phosphatidylcholine, di-palmitoyl			
Phosphatidylethanolamine			
Phosphorylcholine $\cdot HCl$			
Phosphorylethanolamine			
Sphingomyelin			
Tween 40 (ml/liter)			
Tween 80		5	20





<u>Polyamines</u> (mg/liter)			
Putrescine			
Spermine			
Spermidine			
<u>Proteins</u> (mg/liter)			
Bovin serum albumin			
<u>Undefined components</u> (mg/liter)			
Brain extract (ml/l)			
Brain heart infusion			
Fetal bovine serum (ml/l)			
Lactalbumin hydrolysate			
Liver digest			
Peptone			
Phytone			
Tryptone			
Yeast autolysate	2,000		
Yeast extract (Kuske) (ml/l)			
<u>Other components</u> (mg/liter)			
N-acetyl-D-Glucosamine			
Polyvinylpyrrolidone K90			
Penicillin G ( $10^5$ U/ml)			
Phenol red (0.2%) (ml/l)		20	20
pH			
mOsm			



Table 21. MLO Cultivation Media.

Component	PL-3 PL-3S, 3SY	PL-1M	
<u>Inorganic salts and buffers (mg/liter)</u>			
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	50	6	
$\text{CoCl}_2 \cdot 2\text{H}_2\text{O}$	0.01	0.02	
$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$	0.2	1.0	
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$		0.8	
$\text{FeNH}_4$ -citrate	6		
$\text{H}_3\text{BO}_4$	$5 \times 10^{-4}$	0.02	
Hepes		7,100	
KCl	1,000	580	
$\text{KH}_2\text{PO}_4$	1,000	870	
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$			
MgO	1,000	60	
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	2,000	60	
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$			
$\text{MnSO}_4$	5	1.0	
NaCl		12	
$\text{NaHCO}_3$	100	100	
$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$			
$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$			
$\text{Na}_2\text{MoO}_4$	0.01	0.02	
$(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$			
$\text{ZnCl}_2 \cdot 4\text{H}_2\text{O}$	0.01	0.01	



Amino Acids (mg/liter)			
L-Alanine	3,500	250	
$\beta$ -Alanine	140		
L-Arginine·HCl	3,600	50	
L-Asparagine	4,600	500	
L-Aspartic acid	11,600	250	
L-Cysteine·HCl	140	50	
L-Cystine·2HCl			
GABA	70		
L-Glutamic acid	26,000	200	
L-Glutamine	15,400	500	
Glycine	350	50	
L-Histidine	1,050	150	
L-Hydroxyproline	70		
L-Isoleucine	2,300	520	
L-Leucine	2,300	400	
L-Lysine·HCl	2,600	50	
L-Methionine	200	50	
L-Phenylalanine	2,900	330	
L-Proline	200	110	
L-Serine	11,200	1,500	
L-Threonine	5,000	360	
L-Tryptophan	1,400	5	
L-Tyrosine	1,250	40	
L-Valine	4,100	600	





Nucleic acid precursors (mg/liter)			
DNA, herring sperm	10	10	
RNA, torula yeast	2.5	2.5	
Adenine	25	25	
Adenosine	50	50	
Adenosine monophosphate	5	5	
Adenosine triphosphate	2.5	2.5	
Cytidine	25	25	
Cytidine monophosphate	5	5	
Cytidine triphosphate	2.5	2.5	
Guanine	50	50	
Guanosine	100	100	
Guanosine monophosphate	5	5	
Guanosine triphosphate	2.5	2.5	
Hypoxanthine	25	25	
Inosine	50	50	
Inosine monophosphate	10	5	
Thymidine	50	50	
Thymidine monophosphate	5	5	
Thymidine triphosphate	2.5	2.5	
Thymine	25	25	
Uracil	25	25	
Uridine	50	50	
Uridine monophosphate	5	5	
Uridine 5'-triphosphate	2.5	2.5	
Xanthine	25	25	
Xanthine monophosphate	5	5	
2'-Deoxyadenosine	50	50	
2'-Deoxyadenosine monophosphate	5	5	
2'-Deoxycytidine	25	25	



2'-Deoxycytidine monophosphate	5	5	
2'-Deoxyguanosine	50	50	
2'-Deoxyguanosine monophosphate	5	5	
2'-Deoxyuridine monophosphate	5	5	
5-Methyldeoxycytidine			
Phosphoribosyl pyrophosphate	50	50	
D-(-)-Ribose	250	250	
Ribose-1-phosphate	50	50	
2'-Deoxy-D-ribose	250	250	



<u>Cofactors (mg/liter)</u>			
Coccarboxylase	5	2.25	
Coenzyme A	5	2.25	
Flavin adenine dinucleotide	5	2.25	
Nicotinamide adenine dinucleotide	5	2.25	
Nicotinamide adenine dinucleotide phosphate	5	2.25	
<u>Vitamins, Reducing agents (mg/liter)</u>			
Ascorbic acid	20	10	
p-Aminobenzoic acid	0.05	0.05	
d-Biotin	0.5	0.5	
Calciferol			
Calcium pantothenate	0.01		
Choline chloride			
Folic acid	0.2	0.1	
Glutathione (reduced)	25	25	
i-Inositol			
Menadione			
Niacin (Nicotinic acid)	0.5	0.5	
Niacinamide	0.2	0.2	
Pyridoxal·HCl			
Pyridoxal-5-phosphate	0.5	0.5	
Pyridoxine·HCl	0.5	0.5	
Riboflavin	0.5	0.5	
Thiamine·HCl	0.4	0.4	
α-Tocopherol phosphate			
Vitamin A			
Vitamin B <sub>12</sub>	0.1	0.1	





Carbohydrates, Sugars, Sugar alcohols, Phosphorylated Sugars, Osmoregulators (mg/liter)

Choline chloride	50	40	
Erythrose	500	400	
Fructose	1,000	730	
Fructose-6-phosphate	400	50	
Glucose	3,000	730	
Glucose-6-phosphate	400	50	
Glycerol-3-phosphate	50	6	
i-Inositol	50	35	
Maltose			
Mannitol	100		
Sodium glucuronate			
Sorbitol			
Sucrose	10,000	22,000	
Trehalose	100		
Xylose	25		

Organic acids (mg/liter)

Acetic acid	150	65	
Fumaric acid	25		
$\alpha$ -Ketoglutaric acid	250	170	
Malic acid	150	100	
Mevalonic acid	100	65	
Oxalacetic acid	10	7	
Oxalic acid	50	110	
Pyruvic acid	25	17	
Shikimic acid	50	35	
Succinic acid	25		



Lipids (mg/liter)			
Feldlaufer Sterols	12		
Campesterol	0.5	0.24	
Cholesterol	2	2.1	
Sitosterol	3	0.24	
Stigmasterol	1	0.24	
Linoleic acid	0.5	0.24	
Oleic acid	10	1.1	
Palmitic acid	10	1.1	
Glycerol	100	75	
DL- $\alpha$ - Glycerophosphate $\cdot 6H_2O$	1	0.24	
Phosphatidic acid	1	0.24	
Phosphatidylcholine	1.5	0.45	
Phosphatidylcholine, di-oleoyl	1	0.24	
Phosphatidylcholine, di-palmitoyl	1	0.24	
Phosphatidylethanolamine	1	0.24	
Phosphorylcholine $\cdot HCl$	1		
Phosphorylethanolamine	1	0.24	
Sphingomyelin	1.5	0.45	
Tween 40 (ml/liter)	0.1	0.02	
Tween 80 (ml/liter)	0.1	0.02	



<u>Polyamines/Ureides</u> (mg/liter)			
Canavanine	5		
Citrulline	5		
Putrescine	0.5	0.1	
Spermine	10	2.5	
Spermidine	2	0.1	
<u>Proteins</u> (mg/liter)			
Bovin serum albumin	12,000	3,500	
<u>Undefined components</u> (mg/liter)			
Brain extract (ml/l)			
Brain heart infusion			
Lactalbumin hydrolysate			
Liver digest			
Peptone, proteose	3,000		
Phytone	3,000	5,000	
Serum, fetal bovine	PL-3S = 50 ml		
Serum, newborn calf		50 ml	
Tryptone	1,000		
Yeast autolysate		1,000	
Yeast extract (25%)	PL-3SY = 100 ml		
<u>Other components</u> (mg/liter)			
N-acetyl-D-Glucosamine			
Polyvinylpyrrolidone K90			
Ampicillin (25 mg/ml)		2 ml	
Penicillin G ( $10^5$ U/ml)	10		
Phenol red (0.2%) (ml/l)	10		
pH	7.0	7.1	
mOsm	>1,000	519	



Table 22A. MLO Cultivation Media.

Component	PL3-1	PL3-2	PL3-3
<u>Inorganic salts and buffers (mg/liter)</u>			
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	4.7	4.7	4.7
$\text{CoCl}_2 \cdot 2\text{H}_2\text{O}$	0.009	0.009	0.009
$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$	0.2	0.2	0.2
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$			
$\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$			
$\text{FeNH}_4$ -citrate	5.6	5.6	5.6
$\text{H}_3\text{BO}_4$	0.0005	0.0005	0.0005
Hepes			
KCl	935	935	935
$\text{KHCO}_3$	75	75	75
$\text{KH}_2\text{PO}_4$	935	935	935
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$			
MgO	455	455	455
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	455	455	455
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$			
$\text{MnSO}_4$	4.7	4.7	4.7
NaCl (from peptones)	168	168	168
$\text{NaHCO}_3$			
$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$			
$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$			
$\text{Na}_2\text{MoO}_4$	0.0001	0.0001	0.0001
$(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$			
$\text{ZnCl}_2 \cdot 4\text{H}_2\text{O}$	0.009	0.009	0.009





Amino Acids (mg/liter)			
L-Alanine	34	204	408
$\beta$ -Alanine			
L-Arginine·HCl	19	114	228
L-Asparagine	112	672	1,344
L-Aspartic acid	104	624	1,248
L-Cysteine·HCl	8	48	96
L-Cystine·2HCl	2	12	24
L-Glutamic acid	176	1,056	2,112
L-Glutamine	244	1,464	2,928
Glycine	8	48	96
L-Histidine	16	96	192
L-Hydroxyproline	8	48	96
L-Isoleucine	54	324	648
L-Leucine	52	312	624
L-Lysine·HCl	48	288	576
L-Methionine	16	96	192
L-Phenylalanine	42	252	504
L-Proline	24	144	288
L-Serine	208	1,248	2,496
L-Threonine	59	354	708
L-Tryptophan	29	84	150
L-Tyrosine	16	95	190
L-Valine	67	402	804



Nucleic acid precursors (mg/liter)			
DNA, herring sperm	24	24	24
RNA, torula yeast	24	24	24
Adenine	23	23	23
Adenosine	46	46	46
Adenosine monophosphate	4.6	4.6	4.6
Adenosine triphosphate	2.3	2.3	2.3
Cytidine	23	23	23
Cytidine monophosphate	4.6	4.6	4.6
Cytidine triphosphate	2.3	2.3	2.3
Guanine	47	47	47
Guanosine	93	93	93
Guanosine monophosphate	4.6	4.6	4.6
Guanosine triphosphate	2.3	2.3	2.3
Hypoxanthine	23	23	23
Inosine	46	46	46
Inosine monophosphate	9.2	9.2	9.2
Thymidine	46	46	46
Thymidine monophosphate	4.6	4.6	4.6
Thymidine triphosphate	2.3	2.3	2.3
Thymine	23	23	23
Uracil	23	23	23
Uridine	46	46	46
Uridine monophosphate	4.6	4.6	4.6
Uridine 5'-triphosphate	2.3	2.3	2.3
Xanthine			
Xanthine monophosphate	4.6	4.6	4.6
2'-Deoxyadenosine	46	46	46
2'-Deoxyadenosine monophosphate	4.6	4.6	4.6
2'-Deoxycytidine	23	23	23



2'-Deoxycytidine monophosphate	4.6	4.6	4.6
2'-Deoxyguanosine	93	93	93
2'-Deoxyguanosine monophosphate	4.6	4.6	4.6
2'-Deoxyuridine monophosphate	4.6	4.6	4.6
5-Methyldeoxycytidine			
Phosphoribosyl pyrophosphate	47	47	47
D-(-)-Ribose	280	280	280
Ribose-1-phosphate	47	47	47
2'-Deoxy-D-ribose	280	280	280





<u>Cofactors (mg/liter)</u>			
Coccarboxylase	0.9	0.9	0.9
Coenzyme A	0.9	0.9	0.9
Flavin adenine dinucleotide	0.9	0.9	0.9
Nicotinamide adenine dinucleotide	0.9	0.9	0.9
Nicotinamide adenine dinucleotide phosphate	0.9	0.9	0.9
<u>Vitamins, Reducing agents (mg/liter)</u>			
Ascorbic acid	18.7	18.7	18.7
p-Aminobenzoic acid	0.5	0.5	0.5
d-Biotin	0.5	0.5	0.5
Calciferol			
Calcium pantothenate	0.01	0.01	0.01
Choline chloride			
Folic acid	0.2	0.2	0.2
Glutathione (reduced)	28	28	28
i-Inositol			
Menadione			
Niacin (Nicotinic acid)	0.5	0.5	0.5
Niacinamide	0.2	0.2	0.2
Pyridoxal·HCl			
Pyridoxal-5-phosphate	0.5	0.5	0.5
Pyridoxine·HCl	0.5	0.5	0.5
Riboflavin	0.5	0.5	0.5
Thiamine·HCl	0.4	0.4	0.4
α-Tocopherol phosphate			
Vitamin A			
Vitamin B <sub>12</sub>	0.1	0.1	0.1



Table 22B. MLO Cultivation Media.

Component	PL3-4	PL3-5	PL3-P
<u>Inorganic salts and buffers (mg/liter)</u>			
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	4.7	4.7	4.7
$\text{CoCl}_2 \cdot 2\text{H}_2\text{O}$	0.009	0.009	0.009
$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$	0.2	0.2	0.2
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$			
$\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$			
$\text{FeNH}_4$ -citrate	5.6	5.6	5.6
$\text{H}_3\text{BO}_4$	0.0005	0.0005	0.0005
Hepes			
KCl	935	935	935
$\text{KHCO}_3$	75	75	75
$\text{KH}_2\text{PO}_4$	935	935	935
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$			
MgO	455	455	455
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	455	455	455
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$			
$\text{MnSO}_4$	4.7	4.7	4.7
NaCl (from peptones)	168	168	168
$\text{NaHCO}_3$			
$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$			
$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$			
$\text{Na}_2\text{MoO}_4$	0.0001	0.0001	0.0001
$(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$			
$\text{ZnCl}_2 \cdot 4\text{H}_2\text{O}$	0.009	0.009	0.009



Amino Acids (mg/liter)			
L-Alanine	204	204	204
$\beta$ -Alanine			
L-Arginine·HCl	1,114	114	114
L-Asparagine	3,672	672	672
L-Aspartic acid	624	624	624
L-Cysteine·HCl	48	48	48
L-Cystine·2HCl	12	12	12
L-Glutamic acid	1,056	1,056	1,056
L-Glutamine	2,464	1,464	1,464
Glycine	225	48	48
L-Histidine	96	96	96
L-Hydroxyproline	48	48	48
L-Isoleucine	324	324	324
L-Leucine	312	312	312
L-Lysine·HCl	288	288	288
L-Methionine	96	96	96
L-Phenylalanine	252	252	252
L-Proline	144	144	144
L-Serine	1,248	1,248	1,248
L-Threonine	354	354	354
L-Tryptophan	84	84	84
L-Tyrosine	95	95	95
L-Valine	402	402	402



Nucleic acid precursors (mg/liter)			
DNA, herring sperm	24	24	24
RNA, torula yeast	24	24	24
Adenine	23	23	23
Adenosine	46	46	46
Adenosine monophosphate	4.6	4.6	4.6
Adenosine triphosphate	2.3	2.3	2.3
Cytidine	23	23	23
Cytidine monophosphate	4.6	4.6	4.6
Cytidine triphosphate	2.3	2.3	2.3
Guanine	47	47	47
Guanosine	93	93	93
Guanosine monophosphate	4.6	4.6	4.6
Guanosine triphosphate	2.3	2.3	2.3
Hypoxanthine	23	23	23
Inosine	46	46	46
Inosine monophosphate	9.2	9.2	9.2
Thymidine	46	46	46
Thymidine monophosphate	4.6	4.6	4.6
Thymidine triphosphate	2.3	2.3	2.3
Thymine	23	23	23
Uracil	23	23	23
Uridine	46	46	46
Uridine monophosphate	4.6	4.6	4.6
Uridine 5'-triphosphate	2.3	2.3	2.3
Xanthine			
Xanthine monophosphate	4.6	4.6	4.6
2'-Deoxyadenosine	46	46	46
2'-Deoxyadenosine monophosphate	4.6	4.6	4.6
2'-Deoxycytidine	23	23	23





2'-Deoxycytidine monophosphate	4.6	4.6	4.6
2'-Deoxyguanosine	93	93	93
2'-Deoxyguanosine monophosphate	4.6	4.6	4.6
2'-Deoxyuridine monophosphate	4.6	4.6	4.6
5-Methyldeoxycytidine			
Phosphoribosyl pyrophosphate	47	47	47
D-(-)-Ribose	280	420	280
Ribose-1-phosphate	47	47	47
2'-Deoxy-D-ribose	280	420	280



<u>Cofactors</u> (mg/liter)			
Coccarboxylase	0.9	0.9	0.9
Coenzyme A	0.9	0.9	0.9
Flavin adenine dinucleotide	0.9	0.9	0.9
Nicotinamide adenine dinucleotide	0.9	0.9	0.9
Nicotinamide adenine dinucleotide phosphate	0.9	0.9	0.9
<u>Vitamins, Reducing agents</u> (mg/liter)			
Ascorbic acid	18.7	18.7	18.7
p-Aminobenzoic acid	0.5	0.5	0.5
d-Biotin	0.5	0.5	0.5
Calciferol			
Calcium pantothenate	0.01	0.01	0.01
Choline chloride			
Folic acid	0.2	0.2	0.2
Glutathione (reduced)	28	28	28
i-Inositol			
Menadione			
Niacin (Nicotinic acid)	0.5	0.5	0.5
Niacinamide	0.2	0.2	0.2
Pyridoxal·HCl			
Pyridoxal-5-phosphate	0.5	0.5	0.5
Pyridoxine·HCl	0.5	0.5	0.5
Riboflavin	0.5	0.5	0.5
Thiamine·HCl	0.4	0.4	0.4
α-Tocopherol phosphate			
Vitamin A			
Vitamin B <sub>12</sub>	0.1	0.1	0.1



Carbohydrates, Sugars, Sugar alcohols, Phosphorylated Sugars,  
Osmoregulators (mg/liter)

Choline chloride	56	84	56
Erythrose	336	504	336
Fructose	1,120	1,680	1,120
Fructose-6-phosphate	29	29	29
Glucose	3,360	5,040	3,360
Glucose-6-phosphate	374	374	374
Glycerol	112	168	112
Glycerol-3-phosphate	47	47	47
i-Inositol	56	84	56
Mannitol			
Phosphorylribose-1- pyrophosphate	47	47	47
Ribose-1-phosphate	47	47	47
Sodium glucuronate			
Sorbitol			
Sucrose	33,600	50,400	33,600
Trehalose	112	168	112
Xylose			
<u>Organic acids (mg/liter)</u>			
Acetic acid	140	140	140
Fumaric acid	23	23	23
$\alpha$ -Ketoglutaric acid	234	234	234
Malic acid	140	140	140
Mevalonic acid	93	93	93
Oxalacetic acid	9	9	9
Oxalic acid	47	47	47
Pyruvic acid	23	23	23
Shikimic acid	47	47	47
Succinic acid	23	23	23





Lipids (mg/liter)			
Feldlaufer Sterols - mostly spinasterol	16	16	16
Campesterol	0.7	0.7	0.7
Cholesterol	2.7	2.7	2.7
Sitosterol	4.1	4.1	4.1
Stigmasterol	1.4	1.4	1.4
Linoleic acid	0.7	0.7	0.7
Oleic acid	13.5	13.5	13.5
Palmitic acid	13.5	13.5	13.5
Glycerol	112	112	112
DL- $\alpha$ - Glycerophosphate $\cdot$ 6H <sub>2</sub> O	1.4	1.4	1.4
Phosphatidic acid	1.4	1.4	1.4
Phosphatidylcholine	2.0	2.0	2.0
Phosphatidylcholine, di-oleoyl	1.4	1.4	1.4
Phosphatidylcholine, di-palmitoyl	1.4	1.4	1.4
Phosphatidylethanolamine	1.4	1.4	1.4
Phosphorylcholine $\cdot$ HCl			
Phosphorylethanolamine	1.4	1.4	1.4
Sphingomyelin	2.0	2.0	2.0
Tween 40 (ml/liter)	0.14	0.14	0.14
Tween 80 (ml/liter)	0.14	0.14	0.14



<u>Polyamines/Ureides</u> (mg/liter)			
Canavanine	4.7	4.7	4.7
Citrulline	4.7	4.7	4.7
Putrescine	0.5	0.5	0.5
Spermine	9.3	9.3	9.3
Spermidine	1.9	1.9	1.9
<u>Proteins</u> (mg/liter)			
Bovin serum albumin	11,383	11,383	11,383
<u>Undefined components</u> (mg/liter)			
Brain extract (ml/l)			
Brain heart infusion			
Fetal bovine serum (ml/l)			
Lactalbumin hydrolysate			
Liver digest			
Peptone			
Phytone	9,350	9,350	14,350
Proteose peptone	1,870	1,870	1,870
Tryptone	1,870	1,870	1,870
Yeast autolysate			
Yeast extract (Kuske) (ml/l)			
<u>Other components</u> (mg/liter)			
N-acetyl-D-Glucosamine			
Polyvinylpyrrolidone K90			
Penicillin G (10 <sup>5</sup> U/ml)	+	+	+
Phenol red (0.2%) (ml/l)	+	+	+
pH	7.27	7.27	7.23
mOsM	553	512	989



Table 22C. MLO Cultivation Media.

Component	PL3-S	PL3-Y	PL3-SY
<u>Inorganic salts and buffers</u> (mg/liter)			
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	4.7	4.7	4.7
$\text{CoCl}_2 \cdot 2\text{H}_2\text{O}$	0.009	0.009	0.009
$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$	0.2	0.2	0.2
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$			
$\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$			
$\text{FeNH}_4$ -citrate	5.6	5.6	5.6
$\text{H}_3\text{BO}_4$	0.0005	0.0005	0.0005
Hepes			
KCl	935	935	935
$\text{KHCO}_3$	75	75	75
$\text{KH}_2\text{PO}_4$	935	935	935
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$			
MgO	455	455	455
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	455	455	455
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$			
$\text{MnSO}_4$	4.7	4.7	4.7
NaCl (from peptones)	168	168	168
$\text{NaHCO}_3$			
$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$			
$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$			
$\text{Na}_2\text{MoO}_4$	0.0001	0.0001	0.0001
$(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$			
$\text{ZnCl}_2 \cdot 4\text{H}_2\text{O}$	0.009	0.009	0.009



Amino Acids (mg/liter)			
L-Alanine	204	204	204
$\beta$ -Alanine			
L-Arginine·HCl	114	114	114
L-Asparagine	672	672	672
L-Aspartic acid	624	624	624
L-Cysteine·HCl	48	48	48
L-Cystine·2HCl	12	12	12
L-Glutamic acid	1,056	1,056	1,056
L-Glutamine	1,464	1,464	1,464
Glycine	48	48	48
L-Histidine	96	96	96
L-Hydroxyproline	48	48	48
L-Isoleucine	324	324	324
L-Leucine	312	312	312
L-Lysine·HCl	288	288	288
L-Methionine	96	96	96
L-Phenylalanine	252	252	252
L-Proline	144	144	144
L-Serine	1,248	1,248	1,248
L-Threonine	354	354	354
L-Tryptophan	84	84	84
L-Tyrosine	95	95	95
L-Valine	402	402	402





Nucleic acid precursors (mg/liter)			
DNA, herring sperm	24	24	24
RNA, torula yeast	24	24	24
Adenine	23	23	23
Adenosine	46	46	46
Adenosine monophosphate	4.6	4.6	4.6
Adenosine triphosphate	2.3	2.3	2.3
Cytidine	23	23	23
Cytidine monophosphate	4.6	4.6	4.6
Cytidine triphosphate	2.3	2.3	2.3
Guanine	47	47	47
Guanosine	93	93	93
Guanosine monophosphate	4.6	4.6	4.6
Guanosine triphosphate	2.3	2.3	2.3
Hypoxanthine	23	23	23
Inosine	46	46	46
Inosine monophosphate	9.2	9.2	9.2
Thymidine	46	46	46
Thymidine monophosphate	4.6	4.6	4.6
Thymidine triphosphate	2.3	2.3	2.3
Thymine	23	23	23
Uracil	23	23	23
Uridine	46	46	46
Uridine monophosphate	4.6	4.6	4.6
Uridine 5'-triphosphate	2.3	2.3	2.3
Xanthine			
Xanthine monophosphate	4.6	4.6	4.6
2'-Deoxyadenosine	46	46	46
2'-Deoxyadenosine monophosphate	4.6	4.6	4.6
2'-Deoxycytidine	23	23	23



2'-Deoxycytidine monophosphate	4.6	4.6	4.6
2'-Deoxyguanosine	93	93	93
2'-Deoxyguanosine monophosphate	4.6	4.6	4.6
2'-Deoxyuridine monophosphate	4.6	4.6	4.6
5-Methyldeoxycytidine			
Phosphoribosyl pyrophosphate	47	47	47
D-(-)-Ribose	280	280	280
Ribose-1-phosphate	47	47	47
2'-Deoxy-D-ribose	280	280	280



<u>Cofactors</u> (mg/liter)			
Coccarboxylase	0.9	0.9	0.9
Coenzyme A	0.9	0.9	0.9
Flavin adenine dinucleotide	0.9	0.9	0.9
Nicotinamide adenine dinucleotide	0.9	0.9	0.9
Nicotinamide adenine dinucleotide phosphate	0.9	0.9	0.9
<u>Vitamins, Reducing agents</u> (mg/liter)			
Ascorbic acid	18.7	18.7	18.7
p-Aminobenzoic acid	0.5	0.5	0.5
d-Biotin	0.5	0.5	0.5
Calciferol			
Calcium pantothenate	0.01	0.01	0.01
Choline chloride			
Folic acid	0.2	0.2	0.2
Glutathione (reduced)	28	28	28
i-Inositol			
Menadione			
Niacin (Nicotinic acid)	0.5	0.5	0.5
Niacinamide	0.2	0.2	0.2
Pyridoxal·HCl			
Pyridoxal-5-phosphate	0.5	0.5	0.5
Pyridoxine·HCl	0.5	0.5	0.5
Riboflavin	0.5	0.5	0.5
Thiamine·HCl	0.4	0.4	0.4
α-Tocopherol phosphate			
Vitamin A			
Vitamin B <sub>12</sub>	0.1	0.1	0.1





<u>Carbohydrates, Sugars, Sugar alcohols, Phosphorylated Sugars, Osmoregulators (mg/liter)</u>			
Choline chloride	56	56	56
Erythrose	336	336	336
Fructose	1,120	1,120	1,120
Fructose-6-phosphate	29	29	29
Glucose	3,360	3,360	3,360
Glucose-6-phosphate	374	374	374
Glycerol	112	112	112
Glycerol-3-phosphate	47	47	47
i-Inositol	56	56	56
Mannitol			
Phosphorylribose-1-pyrophosphate	47	47	47
Ribose-1-phosphate	47	47	47
Sodium glucuronate			
Sorbitol			
Sucrose	33,600	33,600	33,600
Trehalose	112	112	112
Xylose			
<u>Organic acids (mg/liter)</u>			
Acetic acid	140	140	140
Fumaric acid	23	23	23
$\alpha$ -Ketoglutaric acid	234	234	234
Malic acid	140	140	140
Mevalonic acid	93	93	93
Oxalacetic acid	9	9	9
Oxalic acid	47	47	47
Pyruvic acid	23	23	23
Shikimic acid	47	47	47
Succinic acid	23	23	23



Lipids (mg/liter)			
Feldlaufer Sterols - mostly spinasterol	16	16	16
Campesterol	0.7	0.7	0.7
Cholesterol	2.7	2.7	2.7
Sitosterol	4.1	4.1	4.1
Stigmasterol	1.4	1.4	1.4
Linoleic acid	0.7	0.7	0.7
Oleic acid	13.5	13.5	13.5
Palmitic acid	13.5	13.5	13.5
Glycerol	112	112	112
DL- $\alpha$ - Glycerophosphate $\cdot$ 6H <sub>2</sub> O	1.4	1.4	1.4
Phosphatidic acid	1.4	1.4	1.4
Phosphatidylcholine	2.0	2.0	2.0
Phosphatidylcholine, di-oleoyl	1.4	1.4	1.4
Phosphatidylcholine, di-palmitoyl	1.4	1.4	1.4
Phosphatidylethanolamine	1.4	1.4	1.4
Phosphorylcholine $\cdot$ HCl			
Phosphorylethanolamine	1.4	1.4	1.4
Sphingomyelin	2.0	2.0	2.0
Tween 40 (ml/liter)	0.14	0.14	0.14
Tween 80 (ml/liter)	0.14	0.14	0.14



<u>Polyamines/Ureides (mg/liter)</u>			
Canavanine	4.7	4.7	4.7
Citrulline	4.7	4.7	4.7
Putrescine	0.5	0.5	0.5
Spermine	9.3	9.3	9.3
Spermidine	1.9	1.9	1.9
<u>Proteins (mg/liter)</u>			
Bovin serum albumin	11,383	11,383	11,383
<u>Undefined components (mg/liter)</u>			
Brain extract (ml/l)			
Brain heart infusion			
Fetal bovine serum (ml/l)	47		47
Lactalbumin hydrolysate			
Liver digest			
Peptone			
Phytone	9,350	9,350	9,350
Proteose peptone	1,870	1,870	1,870
Tryptone	1,870	1,870	1,870
Yeast autolysate			
Yeast extract, fresh (ml/l)		47	47
<u>Other components (mg/liter)</u>			
N-acetyl-D-Glucosamine			
Polyvinylpyrrolidone K90			
Penicillin G ( $10^5$ U/ml)	+	+	+
Phenol red (0.2%) (ml/l)	+	+	+
pH	7.28	7.26	7.25
mOsM	508	523	465



Table 23. MLO Cultivation Media.

Component	PL4-1		
<u>Inorganic salts and buffers (mg/liter)</u>			
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	5		
$\text{CoCl}_2 \cdot 2\text{H}_2\text{O}$	0.005		
$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$	0.2		
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$			
$\text{FeNH}_4$ -citrate	6		
$\text{H}_3\text{BO}_4$	0.0005		
Hepes			
KCl	1,250		
$\text{KHCO}_3$	100		
$\text{KH}_2\text{PO}_4$	700		
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$			
MgO			
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	500		
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$			
$\text{MnSO}_4$	10		
NaCl (from peptones)	180		
$\text{NaHCO}_3$			
$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$			
$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$			
$\text{Na}_2\text{MoO}_4$	0.0001		
$(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$			
$\text{ZnCl}_2 \cdot 4\text{H}_2\text{O}$	0.005		





Amino Acids (mg/liter)			
L-Alanine	700		
$\beta$ -Alanine			
L-Arginine·HCl	2,000		
L-Asparagine	3,000		
L-Aspartic acid	1,500		
L-Cysteine·HCl	700		
L-Cystine·2HCl			
L-Glutamic acid	1,500		
L-Glutamine	3,000		
Glycine	500		
L-Histidine	600		
L-Hydroxyproline			
L-Isoleucine	700		
L-Leucine	800		
L-Lysine·HCl	600		
L-Methionine	400		
L-Phenylalanine	600		
L-Proline	700		
L-Serine	2,500		
L-Threonine	700		
L-Tryptophan	200		
L-Tyrosine	200		
L-Valine	900		
Total	22,000		



Nucleic acid precursors (mg/liter)			
DNA, herring sperm	20		
RNA, torula yeast	20		
Adenine	50		
Adenosine	150		
Adenosine monophosphate	10		
Adenosine triphosphate	5		
Cytidine	100		
Cytidine monophosphate	5		
Cytidine triphosphate	2.5		
Guanine	25		
Guanosine	100		
Guanosine monophosphate	10		
Guanosine triphosphate	2.5		
Hypoxanthine	25		
Inosine	50		
Inosine monophosphate	10		
Thymidine	50		
Thymidine monophosphate	5		
Thymidine triphosphate	5		
Thymine	25		
Uracil	50		
Uridine	50		
Uridine monophosphate	5		
Uridine 5'-triphosphate	10		
Xanthine	25		
Xanthosine	50		
Xanthine monophosphate	5		
2'-Deoxyadenosine	100		
2'-Deoxyadenosine monophosphate	5		



2'-Deoxycytidine	100		
2'-Deoxycytidine monophosphate	5		
2'-Deoxyguanosine	100		
2'-Deoxyguanosine monophosphate	5		
2'-Deoxyuridine monophosphate	5		
5-Methyldeoxycytidine			
Phosphoribosyl pyrophosphate	200		
Ribose	750		
Ribose-1-phosphate	100		
Deoxyribose	750		
Deoxyribose-1-phosphate	25		





<u>Cofactors</u> (mg/liter)			
Coccarboxylase	0.5		
Coenzyme A	0.5		
FAD	0.5		
NADH	0.5		
NADH	0.5		
<u>Vitamins, Reducing agents</u> (mg/liter)			
Ascorbic acid	30		
p-Aminobenzoic acid			
d-Biotin	0.5		
Calciferol			
Calcium pantothenate	0.01		
Choline chloride	see Carb.		
Folic acid	0.2		
Glutathione (reduced)	30		
i-Inositol	see Carb.		
Menadione			
Niacin (Nicotinic acid)	0.5		
Niacinamide	0.2		
Pyridoxal·HCl			
Pyridoxal-5-phosphate	0.5		
Pyridoxine·HCl	0.5		
Riboflavin	0.5		
Thiamine·HCl	0.4		
α-Tocopherol phosphate			
Vitamin A			
Vitamin B <sub>12</sub>	0.1		



<u>Carbohydrates, Sugars, Sugar alcohols, Phosphorylated Sugars, Osmoregulators (mg/liter)</u>			
Choline chloride	250		
Erythrose	300		
Fructose	3,000		
Fructose-6-phosphate	50		
Glucose	9,000		
Glucose-6-phosphate	400		
Glycerol	200		
Glycerol-3-phosphate	50		
i-Inositol	100		
Maltose			
Mannitol			
Sodium glucuronate			
Sorbitol			
Sucrose	50,000+?		
Trehalose	2,000		
Xylose			
<u>Organic acids (mg/liter)</u>			
Acetic acid, potassium	100		
Fumaric acid			
$\alpha$ -Ketoglutaric acid	300		
Malic acid	50		
Mevalonic acid			
Oxalacetic acid	100		
Oxalic acid			
Pyruvic acid	150		
Shikimic acid	50		
Succinic acid			



<u>Lipids</u> (mg/liter)			
Feldlaufer Sterols	9		
Campesterol	0.5		
Cholesterol	9		
Sitosterol	1.5		
Stigmasterol	1		
Linoleic acid	1		
Oleic acid	9		
Palmitic acid	10		
Glycerol	see Carb.		
DL- $\alpha$ - Glycerophosphate $\cdot$ 6H <sub>2</sub> O	see Carb.		
Phosphatidic acid			
Phosphatidylcholine	3		
Phosphatidylcholine, di-oleoyl			
Phosphatidylcholine, di-palmitoyl			
Phosphatidylethanolamine			
Phosphorylcholine $\cdot$ HCl			
Phosphorylethanolamine			
Sphingomyelin	20		
Tween 40 (ml/liter)	0.5		
Tween 80 (ml/liter)	0.5		



<u>Polyamines/Ureides</u> (mg/liter)			
Canavanine	7.5		
Citrulline			
Putrescine			
Spermidine	10.0		
Spermine	30.0		
<u>Proteins</u> (mg/liter)			
Bovine serum albumin, efaf	15,000		
<u>Undefined components</u> (mg/liter)			
Brain extract (ml/l)			
Brain heart infusion			
Fetal bovine serum (ml/l)			
Lactalbumin hydrolysate			
Liver digest			
Peptone, Protease	2,000		
Phytone	15,000		
Tryptone	1,000		
Yeast autolysate	3,000		
Yeast extract (ml/l)			
<u>Other components</u> (mg/liter)			
N-acetyl-D-Glucosamine			
Polyvinylpyrrolidone K90			
Penicillin G ( $10^5$ U/ml)	$10^3$ U/ml		
Phenol red (0.1%) (ml/l)	20		
pH	7.3?		
mOsm	550?		





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Table 24. Complex media used in MLO cultivation trials.

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AC20M

200 ml Schneider's Drosophila Medium  
20 ml Medium 199  
10 ml CMRL 1066  
60 ml Fetal bovine serum (Hyclone)  
180 ml Histidine buffer (pH 6.2; = 2.15 g histidine)  
pH = 6.35

BEM1 (from B. Kirkpatrick)

BEM2 (from B. Kirkpatrick)

45 mM Glycine  
45 mM HEPES (pH 7.8, 0.5 M)  
4.5 mM MgCl<sub>2</sub>  
9% Sucrose  
1% Bovine serum albumin  
0.03 M Ascorbic acid  
pH 7.3 (adjusted with KOH)

Blood - Blood agar plates.

BM6 - B. Sears basal extraction medium, unpublished.

C-3G (Modified)

17 g Mycoplasma Broth Base (BBL)  
91 g Sucrose  
10 % Fetal bovine serum (Hyclone)  
11 ml Penicillin-G (705 mg)  
6 ml Phenol red (0.5%, w/v)

CBY

92 ml Chang Medium  
5 ml Pig brain extract (Kirkpatrick)  
3 ml Yeast extract (Kuske)

Chang - This medium is proprietary until published.

DCCM - see Table 1.

DMCMH Medium

2 parts DCCM Medium  
1 part M1D Medium  
2 parts Chang Medium  
1 part H-2 Medium  
2 parts H-1 Medium



Gly-Mg

0.3 M Glycine  
0.03 M  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$   
pH 7.3  
mOsmol 915 (adjusted with Sucrose)

Luria - Luria plates.

M1D - see Table 1.

M1D-SO<sub>3</sub> - M1D & 0.01 M  $\text{Na}_2\text{SO}_3$

MEMEK1 Medium

50 ml DCCM Medium  
1 mM EDTA, disodium  
30 mM MOPS  
4 mM L-Cysteine  
0.3% Polyvinylpyrrolidone  
0.5 ml Spermine  
0.05 Glycine  
0.01  $\text{MgCl}_2$   
0.5% Bovine serum albumin  
10 mM L-Glutamine  
7 g Sucrose  
11 ml 1N KOH  
50 ml Distilled water  
pH 7.1  
mOsmol 915

Milk - Milk agar plates.

MIMEK1 Medium

50 ml DCCM Medium  
0.5 ml Spermine  
5 mM L-Glutamine  
5 mM L-Arginine  
0.2% Bovine serum albumin (Hyclone)  
2.5 mM  $\text{MgCl}_2$   
6.8 g Sucrose  
0.3 ml 2N KOH  
4 ml Distilled water  
pH 7.2  
mOsmol 935

MIMEK1P Medium - MIMEK1 & Penicillin-G

P1 - see Table 2.



Percoll - Chen's isolation media with 40-100% Percoll.

PL-1 - see Table 2.

PL-2 - 2x PL-1

PL-2A - PL-2 plates.

R-1 Medium - This Pasteuria medium is proprietary until published.

SerFree (Serum Free Media)

Serum Free Base:

865 ml Distilled water  
20 g Mycoplasma Broth Base (BBL)  
100 ml Yeast extract (25%)  
5 ml L-Arginine·HCl (42%)  
10 ml Glucose (50%)  
600 mg Penicillin-G (= 1000 U/ml final)  
120 mg Polymyxin B (= 1000 U/ml final)  
20 ml Phenol red (0.1%)  
pH 7.4  
mOsmol 330

SerFree0:

1000 ml Serum Free Base

SerFree1:

1000 ml Serum Free Base  
0.1 ml Tween 80

SerFree2:

1000 ml Serum Free Base  
0.2 ml Tween 80

SerFree4:

1000 ml Serum Free Base  
0.4 ml Tween 80





SerFrac (Serum Fraction Medium)

900 ml Distilled water  
21 g Mycoplasma Broth Base (BBL)  
100 ml Yeast extract (25%)  
10 ml Serum Fraction (Difco)  
5 ml L-Arginine·HCl (42%)  
10 ml Glucose (50%)  
600 mg Penicillin-G (= 1000 U/ml final)  
120 mg Polymyxin B  
20 ml Phenol red (0.1%)  
pH 7.4  
mOsmol 320

Tex2M = Excel400 - D. Lynn medium for Tex2 cell line (proprietary).

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## GENERAL DISCUSSION

The diverse approaches utilized in our studies underscore the many ways that advances in seemingly unrelated scientific areas reinforce and augment one another. Detailed consideration of the problems in cultivating MLOs makes it clear that a general understanding of microbial, and particularly mollicute, physiology will probably be necessary if we are to eventually succeed in cultivating these agents.

It is true that in the past seemingly impenetrable barriers to cultivation of fastidious spiroplasmas have been relatively easily breached, as in the case of Spiroplasma kunkelii (Chen and Liao, 1975; Williamson and Whitcomb, 1975), S. mirum (Tully et al., 1977), the Colorado potato beetle spiroplasma (Hackett and Lynn, 1985), and the Drosophila sex ratio organism (Hackett et al., 1987). In the case of MLOs, although the results presented herein are promising, none of the special procedures that succeeded for the fastidious spiroplasmas, including use of co-cultivation systems and/or anaerobiosis, were successful with MLOs.

In such a case, advances made in basic understanding of mollicute physiology are of special importance. The expertise of D. Pollack was of prime importance in developing a vastly improved picture of intermediate metabolism of the plant phloem/leafhopper-inhabiting spiroplasmas. An understanding of the enzymatic pathways universally present in this taxon, together with work done previously by Pollack's



group on acholeplasmas and mycoplasmas, should lead to vastly improved culture media and cultural conditions for fastidious mollicutes. In Appendix 2, we have given in detail the rationale used in developing the generations of MLO media that are in use today.

Many of the results reported herein have considerable value, even in the absence of MLO cultivation. The development of gene probes, and monoclonal and polyclonal antisera to MLOs, now gives scientists the means to identify MLOs in diseased tissue, and to monitor MLO presence in experiments. Indeed, both systems were used to monitor the MLO cultivation trials reported herein.

Demonstrating again the cross-fertilization of research efforts, work on MLO nucleic acids performed in other laboratories during the tenure of this grant (Lim and Sears, 1989; Sears et al., 1990; Kirkpatrick et al., 1990;) gave impetus and direction to our cultivation trials. These workers showed that MLOs could be placed in the Acholeplasma-Anaeroplasma clade in mollicute phylogenetic classifications. This revelation, of great philosophic interest to microbiologists in general (Woese, 1987), is of the utmost practical significance to agricultural scientists. Now that we know which microbial groups are the closest relatives of MLOs, we have substantial clues as to which sets of physiologies to explore most intensively.

The interfaces described here have already led to the establishment of new collaborations and new working teams on MLOs. Under the auspices of



the International Research Program on Comparative Mycoplasmaology of the International Organization for Mycoplasmaology, a concerted effort is now being made to coordinate the comparative studies of mollicutes from around the world. The immense assemblage of disease conditions (McCoy et. al., 1989) throughout the world demands that the effort be intensified and focused on the vital issues concerning these organisms. Cultivation of the organisms would bring these studies to a new, higher phase of research in which laboratory understanding could be brought to bear in the field to achieve disease management.





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## COOPERATION

Cooperative ties in this project were extensive. The entire research team assembled at the 1988 Vienna and 1990 Istanbul meetings of the International Organization of Mycoplasma. At these meetings, ongoing research was discussed, and many valuable suggestions were exchanged. Cooperation among the United States collaborators was especially intense. Research materials were routinely shipped among the collaborators, or, in the case of Beltsville and Rutgers, hand-carried from one laboratory to the other. Collaborative ties extended to several groups not formally included in the grant. Three laboratories in particular, the plant pathology laboratories at Davis, California (Dr. B. C. Kirkpatrick, UC-Davis, and Dr. D. Golino, ARS) and Michigan State University (Dr. B. Sears), the microbial physiology laboratory of Dr. D. Pollack, Ohio State University, Columbus, and the vector laboratory of L. N. Chiykowski, Canada Agriculture, Ottawa, made major collaborative contributions to the quality and quantity of work reported herein. Research on the Western X-disease MLO was done entirely at Davis by Dr. Hackett and Dr. Kirkpatrick. Dr. Chang prepared special media and made them available for the Davis experiments, and for cultivation trials conducted at Georgia. B. Sears has recently become integrally involved in experiments, providing additional expertise in gene probe detection, and high quality MLO inoculum through plant leaf tip cultures. The final series of cultivation experiments performed at Beltsville were monitored by NJAY-MLO ELISA tests performed at Rutgers University. Finally, the continuing collaboration of D. L. Lynn (Beltsville) was vitally important in providing insect cell cultures.



## MAIN ACHIEVEMENTS

Studies on MLOs resulted in major advances in detection and cultivation protocols:

1. Monoclonal antibodies were produced against several additional MLOs, the agents of Eastern X-disease, and the maize bushy stunt, Paulownia witches broom, Flavescence dorée, ash yellows, and elm yellows MLOs.
2. Polyclonal antibodies were produced, by two widely different strategies, to the the NJAY- and WWB-MLOs.
3. Gene probes were developed to walnut witches' broom and NJAY MLOs.
4. Cultivation technologies developed for MLO cultivation enabled limited cultivation of a fastidious, previously uncultivable nematode pathogen, and could lead eventually to commercial production of a biocontrol agent.
5. An early growth flush of MLOs in cell-free media was demonstrated, giving hope to eventual prospects of continuous cultivation. Factors involved in maintaining membrane stability were identified as important.

Studies on the physiology of another group of plant/insect mollicutes (spiroplasmas) led to vastly improved insight into their adaptive strategies.

Chapters in Volume V of The Mycoplasmas on mollicute detection, mollicute physiology, the corn stunt pathogen, and a detailed listing of MLO occurrences were prepared.





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Appendix 1. Effect of Additives on MLO Growth (Medium to Base Future Media On)		
Beneficial	Neutral	Harmful or Unknown ???
<b>4°C</b> (MIMEK1; PL-1)		
<b>mOsm &gt;500</b> (try various)	Inorganic ions (PL3)	Serum & Yeast Extract ??? (Sears; Hackett)
<b>Sucrose &gt;50g</b> (MIMEK1; PL3-5)	Buffer (PL-1M)	High Tryptone ??? (Hackett)
<b>Choline chloride &gt;55mg</b> (MIMEK1)	Cofactors (PL-3)	
<b>Amino acids &gt;16g</b> (PL3, but consult H-1M for balance)	Vitamins (PL-3)	
<b>BSA</b> (PL3)	Reducing agents (PL-3)	
<b>Polyamines &gt;20mg</b> (PL3)	Phytone (PL3-2)	
<b>Glycerol &gt;110mg</b> (MIMEK1; PL3-5)	pH 7.3	
Glucose/Fructose (MIMEK1; PL3-5)		
Sugar phosphates (PL3-5)		
Erythrose/Trehalose (PL3-5)		
NAPs (PL3)		
Organic acids (PL-1M; with less oxalic acid?)		
Lipids (PL3)		
Peptone (PL3)		
Yeastolate, 3g		
Plant extract, fresh		
Mycoplasma broth base (M1D)		

Note: Conclusions about components in boldface script are more certain than ones in normal script.





## APPENDIX 2. RATIONALE FOR PHLOEM-BASED MEDIA

This series of media utilized the "Golden Elixir Approach," in which a defined base medium was produced and undefined components were added to it. The basic approach in medium formulation in this series involved mimicking sap. A modification of this approach involved the use of components that might be taken up more readily than the analogs present in the phloem. It was considered possible that compounds present in low concentrations might be the most important. Such components are present in sufficient concentration for MLOS because they are present at low levels in the sap as a result of plant metabolism. An example of this approach involved supplementation with erythrose, a readily translocated primer, rather than amino acids that derive biosynthetically from erythrose. In general, sugars (erythrose, fructose, glucose-6-phosphate, ribose), because they are more easily transported, could possibly be more efficacious than amino acids such as phenylalanine, tryptophan, or tyrosine.

In any event, experience with A. laidlawii suggests that glutamine and asparagine are extremely important nutrients. These nutrients can, in some metabolisms, yield  $\text{NH}_3$ , which might then be fixed via the carbamoyl phosphate pathway.

### SPECIFIC MEDIUM COMPONENTS

#### Amino Acids

All amino acids were supplied at a level of at least 5 mg/100 ml. In many cases glutamine and asparagine were supplied in a separate fraction.

Component	Suggested Concn. (mg/100ml)	Notes
L-Alanine	25	Product of photosynthesis; formed from pyruvate; high in oats and barley
Arginine	5	Often low in sap, but moderate in oats and barley; may not be important natural metabolite, but <i>S. citri</i> and <i>S. kunkelii</i> utilize this compound for energy
Asparagine	50	High in apple sap; with glutamine, totals 75% of N; moderate concentrations in oats and barley; direct transfer from xylem; can replace



(17 mg/100ml) yeastolate for  
*Spiroplasma citri*, and is essential for  
*Spiroplasma melliferum*

Aspartic acid	25	Moderate concentration in apple sap; dominant in corn sap; moderate in oats and barley; can be used to form AMP from IMP; D. Pollack has suggested that high aspartate in sap may explain low level of IMP there; a basic building block in the synthesis of asparagine, methionine, threonine, isoleucine, or lysine; because it is highly acidic, it must be preneutralized or the entire amino acid fraction must be neutralized
Cysteine	5	Rare or absent in plant sap, but may be rare because it is so essential that it is constantly consumed. Thus, it may be very important. Formed from serine; possibly essential for <i>Spiroplasma melliferum</i> . In contrast to phloem, it is abundant in xylem
Glutamic acid	20	Occurs in moderate concentration in apple and other saps; high in oats and barley; dominates in corn; a product of photosynthesis, it is a basic building block of glutamine, proline, and arginine.
Glutamine	50	High in apple and other saps, e.g., corn; required by <i>Spiroplasma melliferum</i> ; may act as a glutamate(-) donor to hold H(+) ions during K(+) influx during osmoregulation; may also be a direct source of ATP ( $\text{Glm} + \text{Mg} + \text{ADP} = \text{Glu} + \text{ATP}$ ); directly transferred from xylem
Glycine	5	Low in oats, barley, and other saps; formed from serine; nevertheless, it is a major requirement for certain L-phase variants
Histidine	15	Moderate concentrations in saps, including apple, but low in oats and barley
Hydroxyproline	-0-	Not reported from sap
Isoleucine	52	Abundant in apple and other saps; may not be taken up readily



Lysine	5	Low in many saps, but high in oats and barley; formed from aspartate
Methionine	5	Very low (e.g., oats and barley) or absent in saps; formed from aspartate
Phenylalanine	33	Moderate concentrations in some saps, but low in apple sap; a product of lignin digestion; a building block for tyrosine; some mycoplasmas and acholeplasmas are able to synthesize this amino acid; however, it may not be transported
Proline	11	Low in plant saps; formed from glutamate; gram-positive bacteria import this amino acid to regulate osmolarity, or, in some cases, synthesize it from glutamate; this amino acid is not readily taken up; high in insects; insect parasites often utilize
Serine	150	Very high in saps, including poaceous crops such as corn, oats and barley; one of the first products of photosynthesis, it is a basic building block for cysteine and glycine
Threonine	36	Occurs in moderate concentration in saps, including apple; a building block for isoleucine; transferred directly from xylem
Tryptophan	0.5	Although there is a low content in apple and grain saps, tryptophan is absent in most saps; a product of lignin digestion; interaction with light may produce toxic compounds; uptake of this amino acid may be poor
Tyrosine	4	Low in all saps, including apple, oats and barley; a product of lignin digestion; light intermediated reactions may produce toxic compounds; taken up poorly
Valine	60	Occurs in moderate concentrations in apple and other saps; direct transfer from xylem

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\*Concentrations in the phloem are usually in the 1,000 mg range, although some may be as high as 42,000 mg. Some important values are 520 (Ricinus), 3,000 (Vigna), and 10,000 (cucurbits). Phloem sap is also abundant in metabolic precursors of amino acids, e.g., glutamate, aspartate, serine,







and in the subprecursors phenylalanine and threonine. It is also high in the 3 amino acids formed from pyruvate, i.e., alanine, valine, and leucine, and moderate in histidine (which is formed from ribose-5-phosphate).

## Buffers

Buffers are important in optimizing yields of mollicutes. HEPES seems to supply stronger buffering capacity than  $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ ,  $\text{KH}_2\text{PO}_4/\text{NaOH}$ , or Tris-HCl. Spiroplasma yields are generally, but not always higher in the HEPES buffer; however, it is not known whether the growth curves are steeper in non-HEPES buffered media. Concentration of buffer is important; higher or lower concentrations are inhibitory.

Component	mg/100ml	Notes
HEPES	1500	Buffers media from effects of acid production; inhibits protective effect of $\text{Mn}^{++}$ /bicarbonate
$\text{NaHCO}_3$	10	Should be furnished as a separate fraction as a primer; terrestrial animals are high, aquatic animals are low in bicarbonate; with $\text{Mn}^{++}$ , protects Lactobacillus from peroxide; inhibited by HEPES, inorganic phosphate, PPI; stimulated by amino acids

## Cofactors

Cofactors are nonessential for *S. melliferum* and *S. floridicola* if nicotinic acid and riboflavin are provided. In these variations, quantities similar to those used in previous defined media, e.g., H-1 were chosen.

Components	mg/100ml	Notes
Coccarboxylase	0.45	Coenzyme for aldehyde transfer, decarboxylation of keto acids and formation or degradation of ketols
Coenzyme A	0.45	Carrier of acyl groups, e.g., in fatty acid oxidation, synthesis
FAD	0.45	Electron transfer
NADH	0.45	Electron transfer; not toxic; not transported by acholeplasmas; a necessary component for the strain of Mycoplasma fermentans



NADPH	0.45	Lipid biosynthesis and electron transfer; probably not toxic; not transported by acholeplasmas
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**Emulsifiers and stabilizers**

Component	mg/100 ml	Notes
Bovine serum albumin	1200	1200 mg/100ml in H-1 spiroplasma medium
Coconut milk (see undefined)		Protects plant membranes from phospholipases
Polyamines (see polyamines)		
Serum (see redox)		Provides lipids; functions in lipid transport
Tween 40 (palmitate)	0.01ml	0.1 ml/l in CC494 and LD82
Tween 80 (oleate)*	0.04ml	0.04% recommended for A. florum; 0.01 ml/100ml in CC494, H-1, LD82 spiroplasma media

Classical acholeplasmas and A. axanthum use 0.01% [0.01 ml/100 ml] Tween 80 (Sigma). A. florum requires 0.04% Tween 80 (with 0.5% albumin). 0.1% Tween 80 is toxic to most acholeplasmas, but some strains can tolerate up to 0.06%. Oleic acid could not substitute for Tween 80. Some sterol-requiring strains are sensitive to fatty acids.

**Hormones**

Any culture attempt using a plant rationale must take hormones into account. Although it is unlikely that MLOs utilize hormones per se, they may utilize certain metabolites (e.g., tryptophan, acetate, isopentenyl P*Pi*, adenine, geranylgeranyl P*Pi*) that are needed by plants to make hormones or other products (cholesterol or carotenoids). In either case, plant hormone precursor depletion would likely occur. Ruminant Lactobaccilus spp. that are pneumotoxic produce 3-methylindole from IAA, anaerobically. Some Clostridia, Pseudomonas and Rhizobium species also metabolize hormones or their metabolites. Tween 80 is removed from the medium when testing IAA metabolism. Pyruvate acts as a Schiff base cofactor (rather than biotin, etc.). Some important intermediates are: tryptophan -> auxin; acetate -> isopentenyl P*Pi* -> geranyl P*Pi* -> cholesterol -> alkaloids; geranyl P*Pi* -> geranylgeranyl P*Pi* -> gibberillin; geranylgeranyl P*Pi* -> carotenoids; acetate -> isopentenyl P*Pi* + adenine -> cytokinins

Component	Notes
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Absciscic acid  $10^{-2}$  mg/100ml in Ricinus sap

Auxin Plant cell culture media have  $10^{-7}$  to  $10^{-6}$  M indole-3-acetic acid, indole-3-butyric acid or  $\alpha$ -naphthaleneacetic acid;  $11 \times 10^{-4}$  mg/100ml in Ricinus sap; 0.02% IAA used for ruminal lactobacilli

Cytokinin Plant cell culture media has  $10^{-7}$  to  $10^{-5}$  M benzyladenine or N6-isopentenyladenine;  $11 \times 10^{-4}$  mg/100ml in Ricinus sp.

Giberellin Prepared fresh for plant cell culture media at  $10^{-8}$  to  $10^{-6}$  M;  $2 \times 10^{-4}$  mg/100ml in Ricinus sap

## Inorganics

### Suggested Medium Additives

Major Component	mg/100ml	K+	Na+	Mg++	Ca++	Cl-	P04=	S04=
KCl*	120	63				57		
NaCl**	2		1			1		
KH <sub>2</sub> PO <sub>4</sub> ***	100	28					70	
MgSO <sub>4</sub> .7H <sub>2</sub> O****	36			3				15
CaCl <sub>2</sub> .2H <sub>2</sub> O*****	1				0.3	0.4		

Trace Component	mg/100ml	Zn++	Mn++	Fe++	Cu++	Co++	Mo++	B
ZnSO <sub>4</sub> .7H <sub>2</sub> O	0.8	0.2						
MnSO <sub>4</sub> .H <sub>2</sub> O	0.15		0.05					
FeSO <sub>4</sub> .7H <sub>2</sub> O	0.7			0.14				
CuCl <sub>2</sub> .2H <sub>2</sub> O	0.1				0.04			
CoCl <sub>2</sub> .6H <sub>2</sub> O	0.002					0.0005		
Na <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O	0.002						0.001	
H <sub>3</sub> BO <sub>3</sub>	0.005							0.001

\* Inhibitory to *A. laidlawii* and *Mycoplasma mycoides* at >300-440 mg/100 ml in some studies, but in other studies *M. gallisepticum* and *Acholeplasma laidlawii* grew at concentrations >1040 mg. Can be as high as 370 mg/100ml without inhibitory effects for spiroplasmas.

\*\* Inhibitory to spiroplasmas; may interfere with glycolysis. Provided as trace contaminants of other components.

\*\*\* Essential for certain spiroplasmas. Growth throughout range of 1-100 mg/100ml tested, with a tentative optimal at 7-34 mg.





\*\*\*\* 300 mg/100ml is inhibitory to mollicutes. 185 mg/100ml in M1D is needed for spiroplasmas. Can be supplied as Mg oxide (artificial seawater); can assist in anionic adjustment if  $\text{SO}_4^{2-}$  is too high.

\*\*\*\*\* 64 mg/100ml is inhibitory to spiroplasmas. Not required for spiroplasmas.

### Ion Importance

$\text{Ca}^{++}$  - membrane stability; exclusion important for sieve element formation; not required by plant spiroplasmas; may regulate virulence genes

$\text{Cl}^-$  - second largest anion in sap

$\text{Fe}^{++}$  - required for catalase (but this enzyme is lacking in clostridia and mollicutes; may be required by NAD dehydrogenase in *A. laidlawii*)

$\text{Co}^{++}$  - endo- and exonuclease poison; value unclear

$\text{Cu}^{++}$  - needed by NAD dehydrogenase (see  $\text{Fe}^{++}$ )

$\text{K}^+$  - osmoregulation; ATP pump; principal cation in phloem; important; can be supplemented by adding  $\text{K}_2$  or  $\text{KH}_2\text{PO}_4$  to avoid problems with  $\text{Cl}^-$

$\text{Mg}^{++}$  - membrane stability; required by some spiroplasmas

$\text{Mn}^{++}$  - with bicarbonate, disproportionates  $\text{H}_2\text{O}_2$ ; may protect cells in absence of catalase (e.g. *Lactobacillus*); greatly augmented by presence of amino acids; inhibited by HEPES, inorganic phosphate, PPI

$\text{Na}^+$  - antiporter may need ( $\text{Na}^+/\text{H}^+$ ) in *Acholeplasma laidlawii*; may be relatively unimportant or inhibitory

$\text{PO}_4^{3-}$  - when supplied as monobasic salt, causes less divalent cation precipitation; predominant anion in sap, involved in many energy pathways

$\text{SO}_4^{2-}$  - sulfur is usually present in organic form in sap

$\text{Zn}^{++}$  - endo- and exonuclease poison; value unclear

Since metals are usually determined by ashing, the amounts may be misleading; in nature, they are probably mostly bound to organic compounds.

### Lipids and Membranes

Except in cucurbits, lipid scarcity is the rule in phloem. Theoretically, mollicute media should contain free sterol, free fatty acids, and ready-made phospholipids. Most of these components are present in balanced proportions in serum; 20% FCS or HS will satisfy most needs. The question is whether MLOs, with their particular phylogenetic history, will prove to be typical mollicutes. One possible failure of MLOs to grow in conventional media is that the lipids supplied may be too rich or





inhibitory for other reasons. One possible approach would be to supply unsaturated fatty acids, but at proportions no more than 5 ug/ml (0.5%). Cocktails are dangerous because of toxicity; even with nonfastidious acholeplasmas, replacement of the serum component by albumin and lipids can present problems. A balanced additive would be: (1) 10 ug/ml oleate, 10 ug/ml palmitate; (2) 10-20 ug/ml sitosterol, spinasterol or cholesterol; (3) phospholipid mixture of sphingomyelin and phosphatidylcholine; and (4) a detoxifying agent such as 1-2% albumin.

Previous work (Kahane and Razin, 1977) is of some guidance, at least for conventional mollicutes. The percentage of cholesterol taken up by *M. hominis* exceeded that of phosphatidylcholine by a factor of 3-5; acholeplasma did not take up phosphatidylcholine; *M. hominis* took up 3x as much cholesterol as acholeplasmas; dispersions with high cholesterol to dipalmitoyl phosphatidylcholine ratios were better cholesterol donors; mycoplasmas cannot hydrolyze cholesteryl esters; cholesterol was required at concentrations of at least 5 ug/ml, but 30 ug/ml was inhibitory to *M. hominis*. The optimal concentration was about 15 ug/ml. There are alternative strategies for supplying lipids. It may be desirable to supply sterols in an aggregated form, e.g., by attaching them to starch (the basis for Dubois' oleic acid medium for *Mycobacterium*), or by use of liposomes, to decrease the toxicity of lipids.

Many media contain albumin. There are some problems with supplying this material, since it is undefined, and because proteases may cleave it in the medium. In defined fractions, it might be desirable to use PVP (in part for supplying fatty acids, but also as an osmotic agent).

Alternative lipids or lipid sources may be advantageous. Some of the possibilities include: (i) aster lipids; (ii) defined lipids or lipid precursors in conjunction with aster lipids; (iii) Celex can replace serum for *Spiroplasma citri*; if this could be used in MLO media, it might provide an additional strategy for reducing the sodium content of media.

### Fatty acids

In phloem, free fatty acids are 1.2-5.2% of lipid fraction, which itself is 0.13-0.54% of dry weight. Concentrations of free fatty acids should be kept at less than 10-15 ug/ml. Short chain length and unsaturation enhance the fluidity of fatty acids and their derivatives; for example, there is a 6.5 C lower melting temperature for palmitic acid (C16) than for stearic acid (C18) (69.6 C). It is possible that high levels of unsaturated fatty acids or carotenoids (from plants) in MLO membranes may cause them to be sensitive to high temperatures.

<u>Component</u>	mg/100ml	Notes
Acetate		Acholeplasmas synthesize lipids from acetate; compared to cholesterol, carotenoids produced from acetate cause low proton leakiness



Caprylic acid		Present in sap
Linoleic acid	0.1	Present in sap; apparently required by some spiroplasmas
Linolenic acid		Present in sap
Oleic acid	0.5	10-15 mg/l in CC494, LD82, H-1 spiroplasma media; required by <i>S. citri</i> ; high oleate/palmitate ratio increases adenylate charge
Palmitic acid	0.5	10-15 mg/l in CC494, LD82, H-1 <i>S. citri</i> does not require this fatty acid, but it does promote growth; <i>Acholeplasma laidlawii</i> incorporates this fatty acid at 37C, but can synthesize it also; 10 ug/ml stimulates growth of <i>M. gallisepticum</i>
Triolein	0.2	Possibly may serve to supply requirements

#### *Sterols and derivatives*

AY-MLO membranes may be very fluid. If so, the fluidity could be due to incorporation of high amounts of unsaturated fatty acids from plants, plasma membranes (cf. tonoplast) have more saturated fatty acids and sterols than unsaturated fatty acids. Many physiological factors are affected by fatty acid amounts and relative compositions. For example, adenylate charge across membranes increase as oleate/palmitate ratio increase. If sterols are to be added to media, there is a wide choice of sterol species to select from. Sterols are specifically supplemented in several spiroplasma media. Concentrations of sterols have ranged from 0-20 mg/l in CC494, LD82, and H-1 spiroplasma media.

Component	Notes
Bile Salts	Though bile salts are harmful to many microbes, microbes are found in guts where bile salts occur. They enhance lipid solubility, but are destructive to many biological membranes. They are utilized by some clostridia, indicating possible linkages of these mollicute ancestors with sterol pathways. They require NAD as a coenzyme, and a pH of 6 - 7.5 as a substrate for <i>C. innocuum</i> .

#### *Steryl esters*

Steryl esters may be found in high levels in plants. For example,, in apple sap, steryl esters (5 mg/100ml), together with sitosterol, were the major lipids. Although Smith (1954) felt steryl esters could substitute for





for sterols, the evidence is ambiguous.

Component	mg/100ml	Notes
Cholesteryl laurate	0.3	0.01 mg/ml may serve as a substitute for cholesterol for some mollicutes
Cholesteryl acetate/ oleate/stearate/palmitate	0	Cannot substitute for cholesterol
Cholesteryl linoleate		With protein, can penetrate membranes

*Free sterols*

Sterols can be prepared from plant sources. In one experiment, 15 g aster (live weight) yielded 11 mg crude total sterols (M. Feldlaufer, unpublished data).

Campesterol	0.05	Comprised 10% of rye sterols
Chlorophyll	0.1	Stabilizes highly unsaturated membranes
Cholesterol	-0-	Supplied by cholesteryl laurate; stigmasterol or ergosterol cannot replace for S. citri; very small in some plant species; enhances ATPase function at lower temperatures.
Sitosterol	0.3	50% of rye sterols; apple sap has 0.04 mg/100ml; S. melliferum can use this sterol to replace cholesterol; may be supplied as sitosteryl acetate (Sigma)
Spinasterol	1.0	Aster lipids are 83% spinasterol; a delta-7-sterol; 42% of alfalfa sterols (M. Feldlaufer, unpublished results)
Stigmasterol	0.3	25% of rye sterols

*Phospholipids and precursors*

Biosynthesis

Phospholipids and precursors are biosynthesized in most organisms:

choline ---> phosphorylcholine ---> phosphatidylcholine  
(a salvage pathway)





glycerol + serine + fatty acid ---> phosphatidylserine  
                   + ethanolamine ---> phosphatidylethanolamine

glycerol + ethanolamine + fatty acids ---> phosphatidylethanolamine  
                   + methionine ---> phosphatidylcholine

Component	mg/100ml	Notes
Choline chloride (see sugars/osmoregulation)		Not associated with coenzymes; building block of phosphoglycerides; synthesized by <i>S. floricola</i>
Glycerol (see sugars)		Precursor for phospholipids
Ethanolamine		Precursor for phospholipids
myo-inositol (see sugars)		Not associated with coenzymes; building block of phosphoglycerides
Methionine (see amino acids)		Precursor for phospholipids
Phosphatidic acid	0.1	Required by <i>S. citri</i>
Phosphatidylcholine	0.5	Present in sap and plasma membrane of plants; resembles sphingomyelin; needed by phloem sieve tubes, but not <i>S. floricola</i>
Di-oleoyl P-choline	0.1	Needed by <i>S. kunkelii</i>
Di-palmitoyl P-choline	0.1	Needed by <i>S. kunkelii</i>
P-tidylethanolamine	0.2	Can replace dipalmitoyl phosphatidylcholine and dioleoyl phosphatidylcholine for <i>S. citri</i> , not for <i>S. kunkelii</i>
Phosphoglycerate	0.1	Present in sap; although it could be important for microbial growth, there is little data on its transport
Phosphorylethanolamine	0.2	Present in sap and plasma membrane
Sphingomyelin		Promotes growth of <i>S. mirum</i>
Carotenoids		

These components are of special interest since MLOs are now known to have  
 been derived from the acholeplasma lineage, which are associated with  
 carotenoids. Biosynthetic pathways are:



acetate ---> mevalonate ---> isopentenyl PPI --->  
 geranylgeranyl PPI ---> carotenoids (light protection)

Component	mg/100ml	Notes
Acetate		(See organic acids)
Acetoacetate		Could possibly be used to replace early steps in biosynthesis
Mevalonate		Can substitute for acetate in acholeplasmas

#### *Sugar Containing Components*

The possible importance of components such as sphingosine, mannose, or sialic acids is unclear.

#### **Nucleic acids**

Component	mg/100ml	Notes
DNA	0.5	Whole DNA is often used in media; it occurs at 0.5 mg/ml in plant sap; possible sources include salmon sperm, aster, or acholeplasmas
RNA	0.15	In plant sap RNA occurs at 0.16 mg/100ml); mycoplasmas have an exo-ds-RNAase

#### *Nucleic acid precursors*

Although some plants have very unusual nucleosides or bases, (e.g., vicin in Vicia spp.), it is unlikely that the unusual bases would be required for MLOS such as AY, which are generalists.

Supplements of 50 mg/l of any base or nucleoside supply all required NAPs for many spiroplasmas (but not for *S. melliferum*), probably because of impurities in the reagents. But, inclusion of all RNA bases or nucleosides improved growth of all spiroplasmas tested. Nucleotide supplements were required for growth of *Mycoplasma fermentans*.

Nucleosides are transported better than nucleotides. Although nucleotides may not be transported by mollicutes, there are exceptions. High concentrations of nucleotides could exert a toxic influence by altering phosphorus metabolism. Although mycoplasma broth bases and yeast extracts have plenty of bases, they are low in nucleosides, so it may be necessary to add these compounds to the elixir formulation. In general, experience suggested that a desirable ratio would be 10 mg nucleoside > 5 mg base > 1 mg monophosphate > 0.7 mg diphosphate > 0.5 mg triphosphate per 100 ml



medium. As much deoxyriboside as riboside would seem optimal.

Component	mg/100ml	Notes
<u>Purines</u>		
ADE	5	Required by <i>M. pneumoniae</i> ; 5-12.5 mg/100ml in wheat, barley; high in Robinia
ADO	10	Acholeplasmas and mycoplasmas utilize this purine; it achieves a level of 3.6-4.5 mg/100ml in CC494, LD82, H-1 spiroplasma media; 10-30 mg/100ml in wheat, barley; very low amounts in dicots
AMP	1	Although some mycoplasmas and ureaplasmas import AMP, it may pose some problems in transport; perhaps best used in low amounts as a primer
ATP	0.5	Occurs in very high levels in most saps (3.5-130 mg/100 ml); 50-90 mg in cereals; 0.5 mg/100 ml would fulfill a primer role
dADO	10	3.6-4.5 mg/100 ml in CC494 and H-1 spiroplasma media
dAMP	1	May be beneficial in a primer role
HPX	5	Potential intermediate ADE ---> GUA
INO	10	Potential intermediate ADE ---> GUA
IMP	1	Potential intermediate ADE ---> GUA; 0.2 mg/100 ml in H-1 spiroplasma media; 3 mg inosine in LD82
XMP	1	<i>S. floricola</i> has an enzyme to convert to GMP
GUA	5	Very hard to solubilize; minimal precursor in <i>M. mycoides</i> ; mollicutes are low G + C; should therefore be lower than A or T
GUO	10	Hard to solubilize; component of CC494, LD82, H-1 spiroplasma media





GMP	1	Some mycoplasmas and ureaplasmas import GMP
GTP	0.5	Occurs in plant sap; 0.5 would serve a function
dGUO*	10	Occurs in H-1 spiroplasma media
dGMP	1	
<u>Pyrimidines</u>		
URA	5	Because spiroplasmas do not have UDE phosphorylase, they may need URA; minimal precursor for M. mycoides
URD	10	In CC494, H-1 spiroplasma media; occurs in cereals, but at very low concentration in dicots
dUMP*	1	Some mycoplasmas and ureaplasmas import monophosphates; potential source of dR-I-P
UDP	1	Precursor of dUDP, dTMP
UTP	0.5	Occurs in plant saps: occurs at 2-4.5 mg/100ml in CC494, LD82, H-1 spiroplasma media; 0.5 mg/100ml as a primer; precursor of CTP
CDE	5	2-4.5 mg/100ml in CC494, LD82, H-1 media; occurs in cereals, but is very low in dicots
CMP	1	
dcMP	1	Some mycoplasmas and ureaplasmas import monophosphates; potential source of dR-l-P
CTP	0.5	Occurs in plant sap; could be used at 0.5 mg/100 ml as a primer
THY	5	Minimal precursor for growth of Mycoplasma mycoides
THD	10	THD was supplied at levels of 3-4.5 mg/100ml in CC494, LD82, H-1
TMP	1	





TTP	0.5	Mycoplasmas scavenge to synthesize TTP; could be supplied at 0.5 mg/100ml as a primer; mollicutes lack a thymidylate synthetase
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#### *Sugar precursors*

PRPP (phosphoribosyl pyrophosphate)	10	Presumably an important component because all mollicutes can utilize it: (base + PRPP ---> NMP)
dRibose	50	Should be supplied at about 50 mg/100ml; should be about 2x to concentration of bases, to drive reaction toward nucleosides; if too much ribose is supplied it would be toxic; although supplied at 4.5-9.0 mg/100ml in CC494 and H-1 spiroplasma media it is not required by spiroplasmas
dRibose-1-Phosphate	10	May be important because mollicutes scavenge it, suggesting a need; however there is little evidence regarding its transport

#### **Organic acids**

Overall, there are low amounts of organic acids in sap. Furthermore, the Krebs cycle appears to be absent from Mollicutes. However, some organic acids have been shown to assist spiroplasma growth.

Component	mg/100ml	Importance
Acetate	10	Should be furnished at 10 mg/100ml as a possible lipid precursor
Acetoacetate	10	Could be used at 10 mg/100ml to bypass acetate for lipid synthesis; can be very toxic to some microorganisms
Citric acid		Present in most saps, abundant in apple sap (50 mg/100ml); important to xylem bacteria; chelates metals; may do more harm than good in medium
Fumaric acid		Sometimes present in sap, but rare in apple sap; although it is a component in M1D, there is no evidence it would be needed



Gluconic acid		Rare in sap
a-Ketoglutaric acid	25	10 mg/100ml improves growth of <i>S. kunkelii</i> , but not <i>S. citri</i> ; present in many saps, but not apple may use up NADH ---> glutamate; $\text{NH}_3 + \alpha\text{-K-ga}$ at pH 8 ---> glutamate in cell; this allows cell to retain $\text{H}^+$ to prevent pH drop even in face of $\text{K}^+$ influx
Malic acid	15	Present in most saps; abundant in apple sap (25 mg/100ml); stored in vacuoles in high concentrations; is a component of M1D spiroplasma media; it has been shown that spiroplasmas possess malate dehydrogenase, so they may be able to use malic acid as an energy/NADH source
Malonic acid		Rare in sap
Mevalonic acid		Used by acholeplasmas as lipid precursor
Oxalic acid		Occurs in some saps; some clostridia are anaerobic oxalate fermenters in rumens; these organisms require 1-2 mM acetate for carbon; oxalate <sup>2-</sup> is antiported into some bacteria (anaerobically), with formate <sup>1-</sup> exported; this generates ATP via a $\text{H}^+$ pump
Oxaloacetic acid	1	Link to amino acids in acholeplasmas and mycoplasmas, but not in spiroplasmas; rare in sap; since aspartate is usually abundant, this may not be needed as a precursor for amino acids; very unstable
Pyruvic acid	25	In some saps; some mutant mycoplasmas can use pyruvate (and not glucose); 440 mg/100 ml inhibitory to spiroplasmas; may be used in Schiff reaction for IAA-metabolizing ruminal lactobacilli; a hydrogen peroxide scavenger
Quinic acid		In some saps



Shikimic acid	5	Acholeplasmas may use to make aromatic amino acids; 4 mg/100ml in apple sap; sink tissues use large amounts of shikimate (for aromatic amino acid synthesis), but they apparently import sugars to get the shikimate skeleton
Tartaric acid		In some saps

### Polyamines and ureides

Polyamines may be used as N-sources, membrane stabilizers, or DNA stabilizers. They are also suspected of being second messengers, intimately associated with cytokinin and giberrellic acid function. These may be free, positively charged polyamines, or phenol conjugates, e.g., the cinnamoyls. The question of whether polyamines are translocated in plants is still unsettled, although they are clearly taken up by suspension cultures. Potential toxicity could be a problem with these components.

Component	mg/100ml	Notes
Agmatine		Precursor of putrescine
Allantoic Acid	1	Occurs at 30 mg/100ml in Acer, Platanus, Aesculus sap
Allantoin	1	Same concentration as allantoic acid in sap
Canavanine	5	Abundant in legume sap (30 mg/100ml) as N-source
Citrulline	5	Abundant in many plant saps (30mg/100ml), e.g., cucurbits, even algae
Ornithine	0.5	Precursor of putrescine
Putrescine	0.1	Moderate concentration in sap (1.5 mg/100ml); precursor of spermine; can be formed from arginine via agmatine; 1.0 mg/100ml may be toxic; impedes DNA repair
Spermine	0.5	Apparently occurs in all microbes; AY-MLO maintenance was promoted by 1.0 mg/100ml
Spermidine	0.3	Apparently occurs in all microbes; precursor of spermine; A. laidlawii





accumulates spermidine, not putrescine  
(Sjostrom and Kenny, 1990, Int. J. Syst.  
Bacteriol. 40:456-461)

S-Adenylmethionine

Precursor of spermidine

## Redox regulators

Intuitively, redox is an important issue. Possible strategies include use of an anaerobic hood, Gaspak and/or reducing agents. A 3-gas, small bottle fermentation system could also be used.

1. Characteristics of clostridial ancestors: *C. ramosum*, *C. tertium* and *C. innocuum* are trehalose-fermenting, gut-inhabiting clostridia; *C. ramosum* is an obligate anaerobe; *C. tertium* is aerotolerant; *C. ramosum* (meso-DAP in cell wall, 26% G + C), *C. innocuum* (no DAP, 43-44% G + C), *C. tertium* (no DAP, 24-28% G + C). *C. ramosum* and *C. innocuum* have B-D-glucosidase (like acholeplasmas, but unlike mycoplasmas), and (unlike most other clostridia) ferment trehalose, galactose, cellobiose, sucrose (i.e., they are ideally suited to microhabitats within plants and/or insects). They (like some other clostridia) also benefit by the presence of bile salts (cholesterol derivatives that help solubilize lipids) in the medium (an early phylogenetic association with cholesterol pathways?). Bile salts are toxic to most microbes.

2. Redox regulation was an important factor for growth of the CPB spiroplasma. *M. neurolyticum* grows better in older media. Anaerobic conditions seem to enhance growth of fastidious mollicutes in poor media.

3. 293 mV across sap membrane (pH 8.0). For each 10-fold decrease in the concentration of oxygen, there is a decrease of 15 mV in the oxygen potential. Methane bacteria need a redox of less than -330mV ( $= 10^{-75}$  that of oxygen in the atmosphere). Some anaerobes are not killed at high potentials, but cannot grow until the medium is more reduced.

4. It is impossible to maintain very low redox potentials simply by removing oxygen; some reducing agents must be used (at minimal levels to reduce toxicity and to simulate the concentrations in most environments). Final concentrations of 0.02-0.05% of reducing agent are noninhibitory. Hungate (1969) used 30 mg/100ml cysteine-HCl and 0.25 ml of H<sub>2</sub>S gas per ml of medium. Or, he used hydrogen, sodium thioglycollate, sodium sulfide, and dithionite. Reazurin (0.0001%) indicates redox  $>-0.042$ . Exposure to oxygen during medium preparation must be avoided or an excessive amount of reducing agent must be added (which must be added just before medium use, and must be kept under oxygen-free conditions). CO<sub>2</sub> is heavy and is easier to use in ridding broth of oxygen (which is not very soluble in water); may need to bubble 30-60 min for heat labile media. Alternatively, growth of another organism can be used to reduce oxygen in medium. Some media, if prepared anaerobically, may be sufficiently reduced to start growth without adding reducing agents, particularly if inoculated heavily. Buffering capacity must be increased to reduce acid fermentation products.



5. Care should be taken in using CO<sub>2</sub> to reduce oxygen, because CO<sub>2</sub> can affect glucose and amino acid uptake by effects on membranes (perhaps by increasing membrane fluidity due to alteration of fatty acids). CO<sub>2</sub> is highly reactive with amines.
6. Ruminal lactobacilli that are pneumotoxic produce 3-methylindole from IAA anaerobically. This is also done by clostridia.
7. Oxygen is a definite requirement for at least some mycoplasmas, presumably to reoxidize NADH.
8. Reducing environment inside host cells causes breakage of disulfide bonds in Chlamydia spp., opening pores to allow nutrient exchange.
9. Whereas air is high in oxygen and low in carbon dioxide, air is scarce in water (carbon dioxide is about the same).

Component	mg/100 ml	Notes
Ascorbic acid	1	10 mg/100ml in plant sap; 30 mg/l in H-1 spiroplasma media
Cysteine-HCl		Hungate (1969) used 0.03% (w/v); not found in plant sap
Glutathione	2.5	25 mg/l in H-1 spiroplasma media; 18-31 mg/100ml in fruit phloem exudate; 31-107 mg/100ml in leaf tissue (animals have 15-310 mg/100ml); these may be low estimates due to water dilution; needed for K <sup>+</sup> entry into cell; prevents oxidation of ascorbic acid; may only function in plants at neutral or acid pH, oxidized at alkaline pH (but phloem has glutathione reductase); functions in amino acid transport; needed in high concentration to total K <sup>+</sup> influx, for osmoregulation; activator of enzymes
Mineral Oil		If this is to be used, a 10-20% overlay is recommended (with N <sub>2</sub> or GasPak spurge)
Serum, fetal bovine (see undefined)		Contains many antioxidants (tocopherol, ascorbic acid, glutathione, ceruloplasmin, transferrin, catalase, superoxide dismutase, glutathione peroxidase); peroxidases in horse serum influence batch quality Sodium thioglycollate all concentrations, from 0.01 up; 100 mg/100ml hurt AY-MLO; at 50 mg/100 survival was best





## sugars and osmoregulators

Component	mg/100 ml	Notes
Choline	5	Needed for glycine-betaine formation for osmoregulation; phospholipid precursor
Erythrose	50	
Fructose	100	As a primer, may be required; 25 mg in apple sap; 1-4% in LD82 and H-1 spiroplasma media
Glucose	100	1-5 mg in apple sap; 1-8% in CC494, LD82, H-1 spiroplasma media; 900 mg/100ml ideal for spiroplasmas; 3% used for ruminal lactobacilli
Glutathione (see redox)		An osmoregulator
Glycerol	10	0.24 ml/l in LD82 spiroplasma medium; <i>M. mycoides</i> may require in lieu of other sugars; can also be an osmoregulator; may be used as phospholipid precursor
m-Inositol	5	10 mg/100ml in plant sap in autumn; 20 mg/100ml in apple sap, also in Asteraceae; apparently, cannot be synthesized, but is useful as a phosphorylated derivative
Mannitol		Abundant in trees, but not in AY-MLO hosts
Raffinose		Aster has <1%
Sorbitol		Abundant in trees, but not in AY-MLO hosts
Sucrose	3,000	Although decline and witches' broom agents appear to occur only in hosts with 15-25% sucrose, aster yellows agents are in asters (1%), <i>Datura</i> (1%), and <i>Solanum</i> (6% sucrose); occurs at levels of 2-3.5% in LD82, H-1 spiroplasma media

An alternative approach is suggested by the work of Maloney et al. (1990), who show importance of anionic sugar (e.g., glucose-6-phosphate) transport by starved *Staphylococcus*,



Streptococcus; the organic anion replaces accumulated Pi in the microbial cell. This step could be useful or necessary as a primer. Glu6P and Gly3P are particularly important, but other sugars may also participate (e.g., 2dG6P, Fruc6P, GluNH<sub>2</sub>P, Ribose5P, MeG6P, Fruc1P, Gal6P, PEP).

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\* Also, see amino acids, lipids, and organic acids.

### Undefined components

The number of undefined components suggested is legion. Some of those to be considered seriously are:

#### *Peptones*

May have a high undesirable fat content; plant-based peptones such as phytone may be most suitable.

Some peptones have almost no Na<sup>+</sup> (e.g., proteose peptone); others have high K<sup>+</sup>/Na<sup>+</sup> ratio (e.g., proteose peptone, neopeptone). A concentration of 3% proteose peptone was used for ruminal lactobacilli.

Most contain significant amounts of Cl<sup>-</sup>, some Fe<sup>++</sup>, ppm toxic inorganics, vitamins.

Acholeplasma parvum required phytone (high K<sup>+</sup>, high in some vitamins) or soybean extract.

#### *Components used for Xylem-limited Bacteria*

Components have included: hemoglobin (10mg), hemin chloride (10-40mg), or FePPi (0.25g), isovitalex (10g), charcoal (2g), soluble starch (2g), Mueller-Hinton agar (38g), cornmeal agar (17g), soytone (2-8g), PPLO broth base (10g), casitone (4-7g), yeast extract (10g), phytone (4g), and trypticase (1g).

#### *Yeast Products*

Yeastolate and/or fresh yeast extract are important for *S. kunkelii* and *S. citri*. The (i) manufacturer and (ii) batch of fresh yeast extract are extremely important. Truly fresh extract, prepared in the laboratory, may be much better than commercial preparations (McGarrrity, 1990). Either product can be inhibitory at higher concentrations.

Low MW (<2000) fractions derived from these undefined products appear to be most important, i.e., NAPs, vitamins, amino acids.





Component	mg/100 ml	Notes
Phytone	500	Required by <i>A. parvum</i>
Serum		Classical component of mycoplasma and spiroplasma media; one major function, that of sterol and lipid supply and transport must be solved in some fashion
Yeast Products		Yeastolate may be less inhibitory than FYE; concentrations determined for spiroplasmas may be of some help, but MLOs may be much more sensitive to inhibitors
<b>Vitamins</b>		
Component	mg/100 ml	Notes
Biotin	0.05	<i>C. butyricum</i> is the only clostridium requiring; required by pyruvate dehydrogenase, which is abundant in acholeplasmas
Folic Acid	0.01	Stimulatory to gut clostridia
Nicotinic Acid	0.05	Fair amount in plant sap, but very low in corn; 0.05 mg/100ml in Murashige and Skoog plant cell formulations; one of 2 vitamins required by spiroplasmas; optimal is 4.87 nM (= $6 \times 10^{-4}$ mg/100 ml) for spiroplasmas
Pantothenic Acid		Present in sap as coenzyme A
PABA		0.01 mg/100 ml in Murashige and Skoog plant cell medium
Pyridoxal-5-PO <sub>4</sub>	0.05	Necessary for transaminases
Pyridoxine HCl	0.05	Occurs in plant sap; 0.05 mg/100ml in Murashige and Skoog plant cell media
Riboflavin	0.05	One of 2 vitamins required by spiroplasmas; optimal is 3.83 nM (= $1.4 \times 10^{-4}$ mg/100ml) for spiroplasmas; stimulatory to gut



clostridia; sensitive to  $\text{Fe}^{++}$ ,  $\text{H}_2\text{O}_2$ ;  
oxidizes substances in light

Thiamine HCl                      0.04      Occurs in plant sap;  
0.04 mg/100ml in Murashige and  
Skoog plant cell media

Vitamin B12                      0.01      Many clostridia utilize

### PHYSICAL FACTORS

#### **Cultivation Systems for Oxygen-sensitive Organisms**

The principal systems considered were (i) use of an anaerobic hood under  $\text{N}_2\text{H}_2$  atmosphere, 0%  $\text{O}_2$ ; (ii) microaerophilic conditions; (iii) use of deep broth or deep agar; and (iv) GasPak and/or 10-20% overlay of mineral oil.

#### **Medium pH**

The pH optima need to be determined empirically. Although pH values of 6.0 to 8.0 have been classically chosen, plant cell cultures are maintained at pH 5.6.

#### *Some pH Values Relevant to MLO Culture*

		Plant Sap	Other Tissues	Animal Cytoplasm
pH		7.2-8.5	Slightly acid	7-8
pH	MLO	MLO	A. laidlawii	
0-6.25		24 h*		
6.5-7.5	Best	48 h	<7.2-7.4 growth ceases	
>7.5		Poor		
7.75-8.0		4 h		
8.5			Best	

\* Data are hours of MLO survival at a pH



Sap pH	Host	MLO Disease		AY	Phyl
		WtBr	Decline		
6.8-7.4	Cucurbita pepo			JAY	
7.1-7.5	Fraxinus spp.	+	+		
7.0 (most)-8.7	Robinia spp.	+			
7.5-8.0	Malus spp.	AP			
8.0	Beta vulgaris	+			
8.0-8.1	Cucurbita maxima			EAY	EuCP

Conclusions are difficult to make.

MLOs may have different pH optima, but it is difficult to imagine that they are profoundly different from spiroplasmas, or that they are extremely exacting, given their residence in phloem, insect gut, hemolymph, and saliva. A neutral to slightly basic pH appears to be adequate.

### Solid Phase Support

Selection of a solid-phase support, such as agar or a filter membrane may be important. Since a single bacterium has a mass of 1.5 pg, there is a total mass of 15 mg in one colony or  $10^7$  CFU/ml. This mass of bacteria is diluted almost 70 times in broth. In semisolid medium, drainage is slow, bacteria are surrounded by metabolites in high concentration.

Agar quality is important. Noble agar, for most other mollicutes, has been employed at 0.8 to 2.4%.

### Temperature

Temperature optima vary among mollicutes, depending on their habitats. Historically, AY organisms have been shown to be heat sensitive, but 28°C appears to be a suitable temperature.







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